THESE DE DOCTORAT

présentée par

Sylvain DEBIEU
Ingénieur ESCOM

pour obtenir le titre de

DOCTEUR DE L'UNIVERSITE DE BOURGOGNE

Mention : Chimie - Spécialité : Chimie Organique

Synthèse *in-situ* de fluorophores organiques - Formation de liaisons covalentes par déclenchement enzymatique et applications en biodétection

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Volume II : Partie expérimentale

Soutenance le 18 octobre 2017 devant la commission d'examen :

FERY-FORGUES Suzanne	Directeur de Recherche CNRS (SPCMIB, UMR 5068)	Rapporteur
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Thèse préparée au sein de l'équipe "Polyamines, Porphyrines, Développements et Applications" (P2DA) de l'Institut de Chimie Moléculaire de Bourgogne (ICMUB) - UMR 6302

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Préambule

Ce second volume regroupe l'ensemble des données expérimentales (protocoles de synthèse et caractérisations physico-chimiques et spectroscopiques des produits obtenus) permettant de reproduire les travaux effectués au cours de cette thèse, ainsi que quelques compléments d'informations auxquels le lecteur pourra se référer si nécessaire. Elle sera organisée en trois parties. Une partie compilera quelques figures et tableaux qui viennent en complément de l'introduction générale. La seconde partie décrit la synthèse et la caractérisation des produits présentés dans le manuscrit et non publiés. Pour les produits disponibles commercialement mais qui ont tout même étaient synthétisés "in house" pour des raisons d'économie ou de gain de temps, seul le protocole de préparation est donné. La troisième partie est composée d'un recueil des parties expérimentales et des fichiers "Supplementary Materials / Supporting Information" des trois publications présentées dans ce manuscrit. Par soucis d'homogénéité, la rédaction en anglais a été privilégiée.

Table of Content

SI.o. Additional table and figures

Fluorophore	Solvent	λ_{max} (nm)	ε ($M^{-1}cm^{-1}$)	λ_{em} (nm)	Φ	$\varepsilon \times \Phi$ ($M^{-1}cm^{-1}$)	Ref
quinine **1**	pH 2	347	5 400	448	0.55	3 000	*1*
phenylalanine **2**	H_2O	260	200	282	0.024	5	*2, 3*
tyrosine **3**	H_2O	275	1 500	303	0.14	210	*2, 3*
tryptophan **4**	H_2O	280	6 300	348	0.13	820	*2, 3*
NADH **5**	H_2O	340	6 200	435	0.019	120	*2, 4*
FMN **6**	H_2O	450	12 200	530	0.25	3 100	*2, 5*
EDANS **7**	H_2O	336	6 100	520	0.27	1 600	*6*
Lucifer Yellow **8**	H_2O	428	12 300	535	0.21	2 600	*7*
pyrene **9**	MeOH	340	43 000	376	0.75	32 000	*8, 9*
4-MU **10**	pH 10	360	17 000	450	0.63	11 000	*10*
AMC **11**	MeOH	351	18 000	430	0.75	14 000	*11*
DAPI **12**	H_2O/DNA	358	21 000	461	0.34	7 100	*8, 12*
Hoechst 33342 **13**	H_2O/DNA	350	45 000	461	0.38	17 000	*8, 12*
NBD **14**	MeOH	465	22 000	535	0.3	7 000	*8*
bimane **15**	pH 7.4	390	5 300	482	0.3	2 000	*13*
Cascade Yellow **16**	MeOH	409	24 000	558	0.56	13 000	*8, 14*
fluorescein **17**	pH 9	490	93 000	514	0.95	88 000	*3, 8*
Rh_{110} **18**	pH 7.5	496	74 000	517	0.92	68 000	*15*
TMR **19**	MeOH	540	95 000	565	0.68	65 000	*11*
SRh_{101} **20**	pH 7	586	108 000	605	0.77	83 000	*8, 11*
naphthofluorescein **21**	pH 9.5	595	44 000	660	0.14	6 200	*16*
SNARF-1 **22**	pH 10	573	44 000	631	0.092	4 100	*17*
propidium **23**	H_2O/DNA	535	5 400	617	0.13	700	*8, 12*
BODIPY-FL **24**	MeOH	505	91 000	511	0.94	86 000	*11*
BODIPY-TR **25**	MeOH	588	68 000	616	0.84	57 000	*11*
Cy3 **26**	pH 7	554	130 000	568	0.14	18 000	*18*
Cy5 **27**	pH 7	652	200 000	672	0.18	36 000	*18*
Cy7 **28**	pH 7	755	200 000	778	0.02	4 000	*18*
IRDye 700DX **29**	H_2O	689	165 000	700	0.14	23 000	*19*
resorufin **30**	pH 9.5	572	56 000	585	0.74	41 000	*20*

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(2) Wolfbeis, O. S. (1985) The fluorescence of organic natural products, in *Molecular Luminescence Spectroscopy: Methods and Applications—Part 1* (Schulman, S. G., Ed.) pp 167-317, Wiley, New York.
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(15) Lavis, L. D., Chao, T.-Y., and Raines, R. T. (2006) Fluorogenic label for biomolecular imaging. *ACS Chem. Biol. 1*, 252-260.
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Table SI.1 - Photophysical properties of fluorophores 1–30[1] and their references introduced page 10 of this manuscript.

[1] L. D. Lavis et R. T. Raines, *ACS Chem. Biol.*, **2008**, (3), 142-155 (SI).

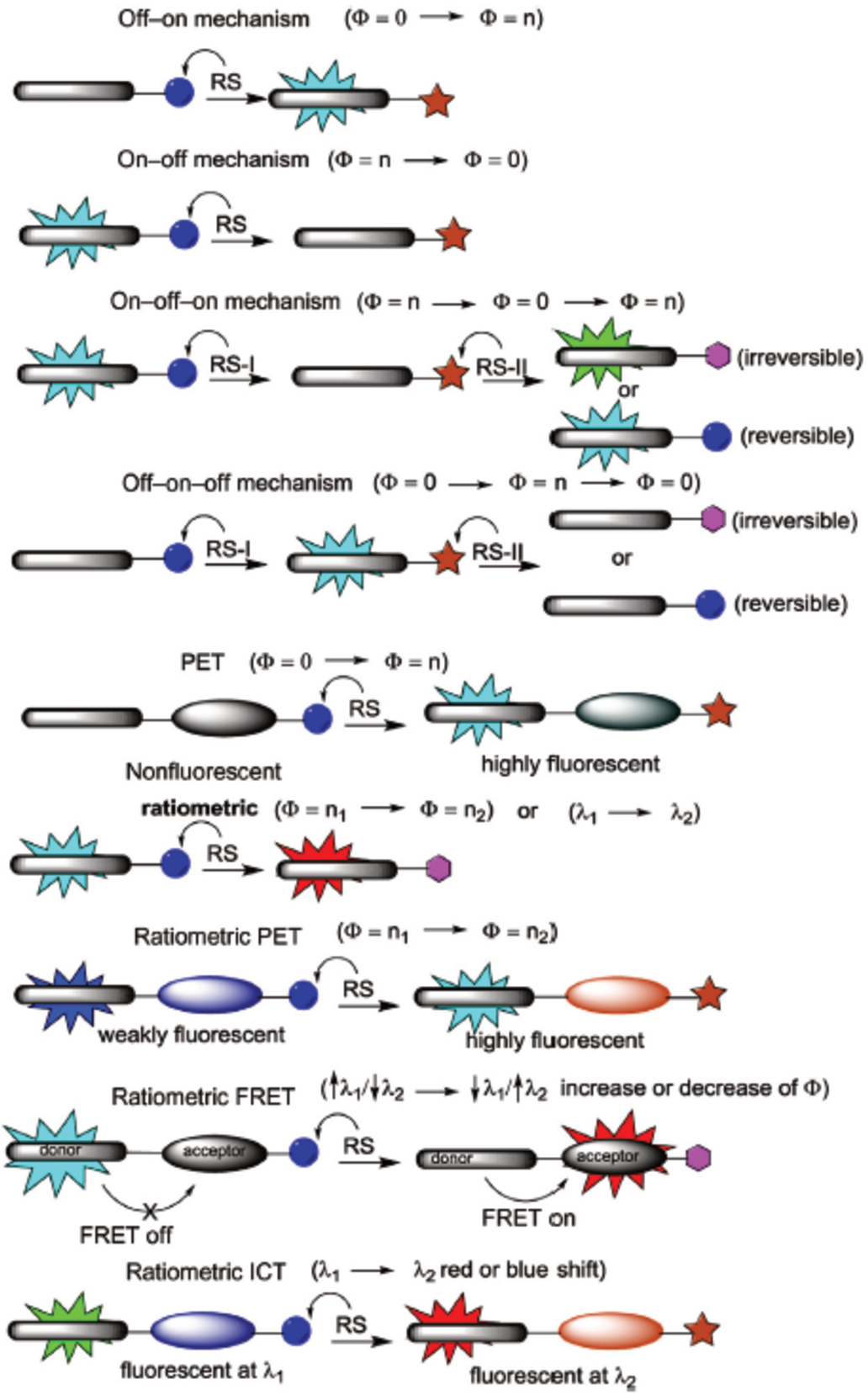


Figure SI.1 - Some general designs of fluorescent probes based on modalities discussed in part 0.1.3. Illustration from Villamena's book chapter[2]. (RS = Reactive Species).

[2] F. A. Villamena, Fluorescence Technique, *Reactive Species Detection in Biology*, Elsevier, **2017**.

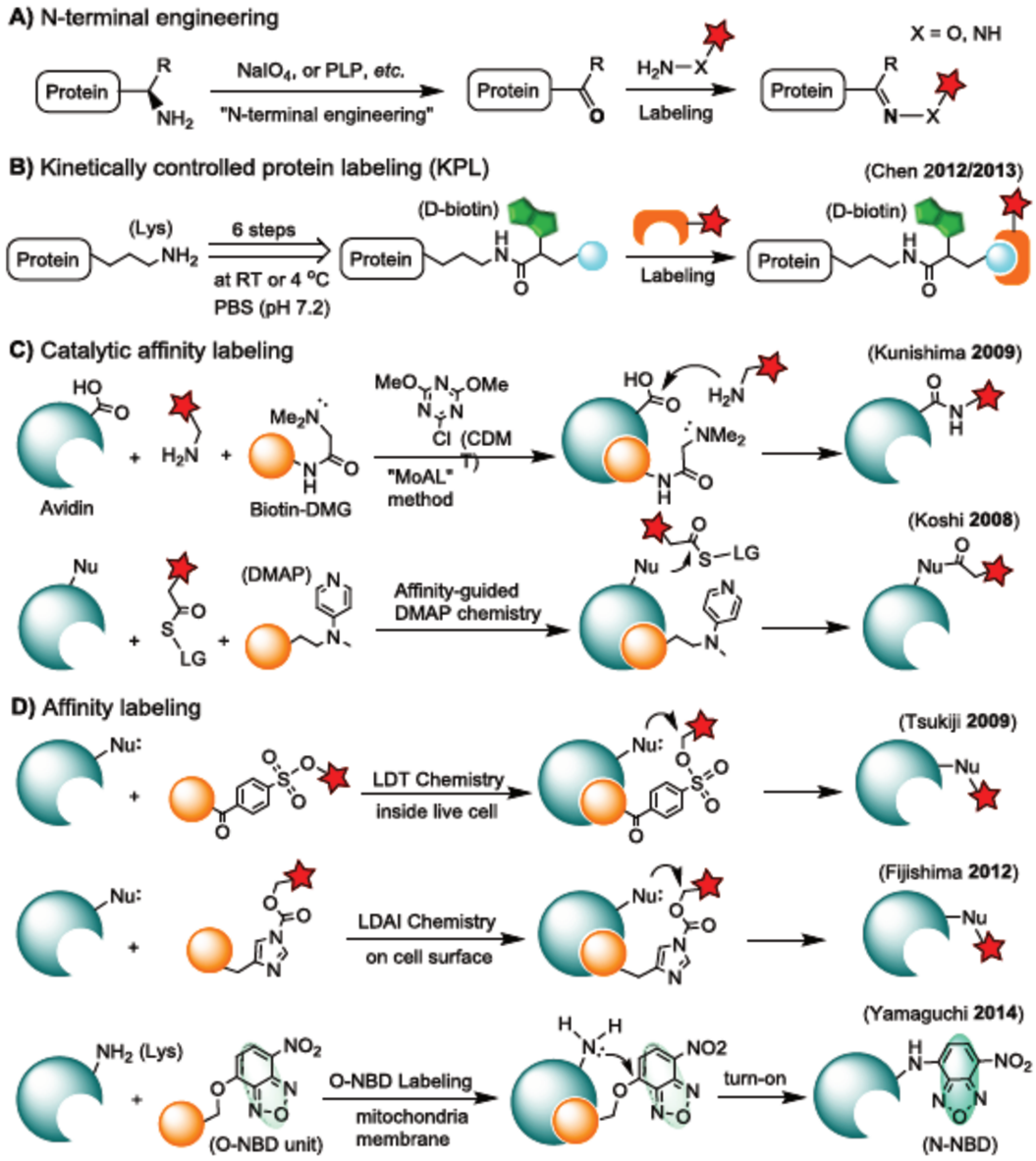


Figure SI.2 - Other methods than photo-labeling used for the chemoselective labeling of native proteins[3]. Illustration from the review [3].

[3] For more information, see: X. Chen and Y.-W. Wu, *Org. Biomol. Chem.*, **2016**, (14), 5417-5439.

SI.1. Unpublished experimental data

SI.1.1. General

Unless otherwise noted, all commercially available reagents and solvents were used without further purification. TLC were carried out on Merck DC Kieselgel 60 F-254 aluminum sheets. The spots were directly visualized or through illumination with UV lamp (λ = 254 / 365 nm) and / or staining with a phosphomolybdic acid solution (4.8% wt. in EtOH). Unless noted otherwise, column chromatography purifications were performed on silica gel (63-200 μm) from Sigma-Aldrich (technical grade) or on automated flash chromatography purification system (Interchim puriFlash™ 430) with puriFlashTM columns (silica gel, 25 μm). Diatomaceous filter-aid was used for several filtrations, using Dicalite® 4158 from Carlo Erba. DCM, MeOH, THF and toluene (HPLC-grade) were dried over alumina cartridges using a solvent purification system PureSolv PS-MD-5 model from Innovative Technology. Anhydrous DMSO and DMF were purchased from Carlo Erba, and stored over activated 3 Å molecular sieves. Anhydrous NMP was purchased from Acros (99.5%, extra dry, AcroSeal®, storage over 3 Å molecular sieves). Petroleum ether (PE) was obtained from Sigma-Aldrich (bp 40-60 °C). The HPLC gradient grade acetonitrile (CH_3CN) was obtained from Biosolve or Carlo Erba. The HPLC gradient grade methanol (MeOH) and absolute EtOH were obtained from Carlo Erba. Formic acid (grade "eluent additive for LC-MS") was provided by Sigma-Aldrich. DIEA (SOL-003) and TFA (peptide grade, SOL-011) were purchased from Iris Biotech GmbH. Phosphate buffer (PB, 100 mM, pH 7.6), and aq. mobile-phases for HPLC were prepared using water purified with a PURELAB Ultra system from ELGA (purified to 18.2 M.cm). PGA (from *E. coli*) was provided by Iris Biotech GmbH (EZ50150, 841 U / mL) and stored at -20 °C. β-Gal (from E. coli, G5635, grade VIII, 500 U / mg, lyophilized enzyme resuspended in ultrapure water), (NTR (from *E. coli*, N9284, 0.1 U / μg, lyophilized enzyme + buffer resuspended in ultrapure water) and NADH were purchased from Sigma-Aldrich and stored at -20 °C.

SI.1.2. Instruments and methods

Freeze-drying steps were performed with an Christ Alpha 2-4 LD plus. Centrifugation steps were performed with a Thermo Scientific Espresso Personal Microcentrifuge instrument. Centrifugation steps (for synthetic purposes) were performed with an Hettich Universal 320 instrument. ^{1}H-, ^{13}C- and ^{19}F-NMR spectra were recorded either on a Bruker Avance 300 or on a Bruker Avance 500 spectrometer. Chemical shifts are expressed in parts per million (ppm) from the residual non-deuterated solvent signal[4]. *J* values are expressed in Hz. IR spectra were recorded with a Bruker Alpha FT-IR spectrometer equipped with an universal ATR sampling accessory. The bond vibration frequencies are expressed in reciprocal centimeters (cm-1). Elemental analyses (C, H, N, S) were perfomed on a Thermo Scientific Flash EA 1112

[4] 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), 2176-2179.

instrument. HPLC-MS analyses were performed on a Thermo-Dionex Ultimate 3000 instrument (pump + autosampler) equipped with a diode array detector (Thermo-Dionex DAD 3000-RS) and a MSQ Plus single quadrupole mass spectrometer (LRMS analyses through ESI mode). Purifications by semi-preparative HPLC were performed on a Thermo-Dionex Ultimate 3000 instrument equipped with a RS Variable Detector (four distinct wavelengths). UV-visible spectra were obtained either on a Varian Cary 50 scan spectrophotometer or on a Jasco V-630 Bio spectrophotometer by using a rectangular quartz cell (Hellma, 100-QS, 45 × 12.5 × 12.5 mm, pathlength 10 mm, chamber volume: 3.5 mL). Fluorescence spectroscopic studies (emission / excitation spectra and kinetics) were performed either with an HORIBA Jobin Yvon Fluorolog spectrophotometer (software FluorEssence™) or with a Jasco FP-8500 spectrofluorometer (software Spectra Manager), using a standard fluorometer cell (Labbox, LB Q, 10 mm). Emission spectra were recorded under the same conditions after excitation at the corresponding wavelength (Ex / Em bandwidth = 5 nm, response = 1s and PMT sensitivity = medium). All fluorescence spectra were corrected.

SI.1.3. High-performance liquid chromatography separations

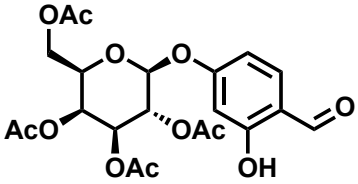
Several chromatographic systems were used for the analytical experiments (HPLC-MS) or the purifications (semi-preparative HPLC) respectively: System A: RP-HPLC-MS (Phenomenex Kinetex C_{18} column, 2.6 μm, 2.1 × 50 mm) with CH_3CN (+ 0.1% FA) and 0.1% aq. FA (pH 3.2) as eluents [linear gradient from 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. UV-visible detection was achieved at 220, 260, 350 and 500 nm (+ diode array detection in the range 220-700 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: semi-preparative RP-HPLC (Thermo BetaBasic-C_{18} column, 5 μm, 30 × 150 mm) with CH_3CN and 0.1% aq. FA (pH 3.2) as eluents [0% CH_3CN (5 min), followed by a gradient of 0% to 20% CH_3CN (15 min), then 20% to 70% CH_3CN (100 min)] at a flow rate of 20.0 mL/min. Quadruple UV detection was achieved at 220, 260, 320 and 370 nm. System C: system B with the following gradient [65% MeOH (5 min), followed by a gradient of 65% to 90% MeOH (20 min), then 90% to 100% MeOH (10 min)]. System D: semi-preparative RP-HPLC (Thermo BetaBasic-C_{18} column, 5 μm, 30 × 150 mm) with CH_3CN and 0.1% aq. TFA (pH 2.0) as eluents [0% CH_3CN (5 min), followed by a gradient of 0% to 30% CH_3CN (10 min), then 30% to 100% CH_3CN (80 min)] at a flow rate of 20.0 mL/min. Triple UV detection was achieved at 220, 260 and 320 nm. System E: semi-preparative RP-HPLC (Thermo BetaBasic-C_{18} column, 5 μm, 150 × 30 mm) with CH_3CN and H_2O as eluents [30% CH_3CN (5 min), followed by a gradient of 30% to 60% CH_3CN (20 min), then 60% to 95% CH_3CN (35 min)] at a flow rate of 20.0 mL/min. Dual UV detection was achieved at 220 and 350 nm. System F: semi-preparative RP-HPLC (Thermo BetaBasic-C_{18} column, 5 μm, 150 × 30 mm) with CH_3CN and 0.1% aq. TFA (pH 2.0) as eluents [1% CH_3CN (10 min), followed by a gradient of 1% to 10% CH_3CN (5 min), then 10% to 40% CH_3CN (45 min)] at a flow rate of 20.0 mL/min. Triple UV detection was achieved at 210, 215 and 220 nm. System G: semi-preparative RP-HPLC (SiliCycle SiliaChrom C_{18} column, 10 μm, 20 × 250 mm) with CH_3CN and 0.1% aq. TFA (pH 2.0) as eluents [2.5% CH_3CN (5 min), followed by a gradient of 2.5% to 15% CH_3CN (5 min), then 15% to 80% CH_3CN (50 min)] at a flow rate of 20.0 mL/min. Quadruple UV-vis

detection was achieved at 220, 260, 350 and 490 nm. System H: semi-preparative RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 10 × 250 mm) with CH_3CN and H_2O as eluents [50% CH_3CN (5 min), followed by a gradient of 50% to 65% CH_3CN (5 min), then 65% to 100% CH_3CN (35 min)] at a flow rate of 4.0 mL/min. Quadruple UV detection was achieved at 220, 260, 300 and 350 nm.

SI.1.4. Synthesis of unpublished compound

Highlighted Compounds of chapter 1

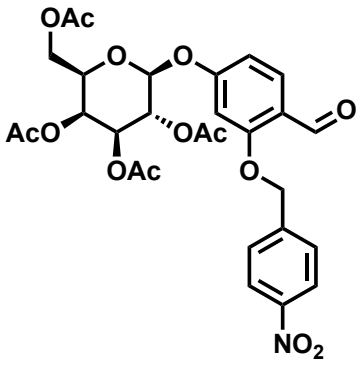
2-Hydroxy-[(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)oxy]-benzaldehyde *(SD090, N° CAS : 103292-13-3)*[5]



A solution of TBAB (75 mg, 0.23 mmol, 0.2 equiv.) in a mixture of H_2O-$CHCl_3$ (1 : 1, v/v, 4 mL) was stirred and heated to 45 °C. 2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl bromide (Sigma-Aldrich, A7562, 500 mg, 1.22 mmol, 1.05 equiv.) was dissolved in $CHCl_3$ (2 mL, named solution A) and 2,4-dihydroxylbenzaldehyde (160 mg, 1.16 mmol, 1 equiv.) was dissolved in deionized water (2 mL) containing $NaHCO_3$ (107 mg, 1.27 mmol, 1.1 equiv.) (named solution B). Then, solutions A and B were simultaneously dropped into the above mixture and was stirred vigorously at 45 °C overnight. Thereafter, the mixture was dissolved in DCM. The organic layer was extracted and washed with brine, dried over anhydrous Na_2SO_4 and concentrated under vacuum. The resulting residue was purified by chromatography on a silica gel column (eluent: heptane-EtOAc, step gradient from 10 : 0 to 5 : 5, v/v) to afford **SD090** as white solid (320 mg, yield 59%).^{1}H NMR (300 MHz, $CDCl_3$): δ = 11.34 (s, 1H), 9.76 (s, 1H), 7.47 (d, *J* 8.4, 1H), 6.61 (dd, *J* 2.1 and 8.4, 1H), 6.56 (d, *J* 2.1, 1H), 5.52-5.45 (m, 2H), 5.14-5.09 (m, 2H), 4.22-4.11 (m, 3H), 2.18 (s, 3H), 2.09 (s, 3H), 2.06 (s, 3H), 2.01 (s, 3H) ppm; HPLC (system A): t_R = 4.7 min, purity = 99% (at 260 nm); LRMS (ESI+): *m/z* 469.1 (100) $[M + H]^+$, calcd for $C_{21}H_{25}O_{12}^+$ 469.1; LRMS (ESI-): *m/z* 467.2 (100) $[M - H]^-$, calcd for $C_{21}H_{23}O_{12}^-$ 467.1.

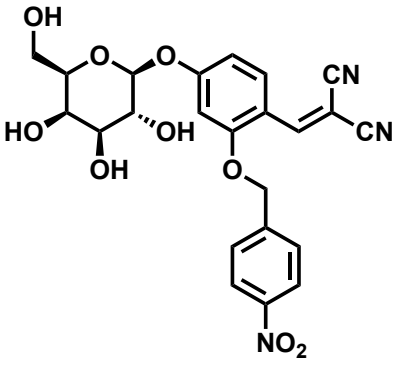
2-(4-Nitrobenzyloxy)-[(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)oxy]-benzaldehyde *(SD093)*

[5] Adapted from: Q. Yan, R. Cao, W. Yi, L. Yu, Z. Chen, L. Ma and H. Song, *Bioorg. Med. Chem. Lett.*, **2009**, (19), 4055-4058.



To a stirred solution of **SD090** (320 mg, 0.68 mmol, 1 equiv.) in Et_2O (3 mL) were sequentially added 4-nitrobenzyl bromide (738 mg, 3.42 mmol, 5 equiv.) and Ag_2O (634 mg, 2.73 mmol, 4 equiv.). The resulting reaction mixture was stirred at RT overnight. Thereafter, the crude was directly purified by chromatography on a silica gel column (eluent: heptane-EtOAc, step gradient from 10 : 0 to 8: 2 to completely remove the large excess of 4-nitrobenzyl bromide (which was recovered) and after from 5 : 5 to 3 : 7, v/v) to afford the desired product **SD093** as white solid (297 mg, yield 66%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 10.41 (s, 1H), 8.28 (d, *J* 8.7, 2H), 7.86 (d, *J* 8.7, 1H), 7.63 (d, *J* 8.7, 2H), 6.69 (dd, *J* 1.8 and 8.7, 1H), 6.60 (d, *J* 2.1, 1H), 5.49-5.44 (m, 2H), 5.25 (s, 2H) 5.14 (t, 1H), 4.23-4.07 (m, 4H), 2.18 (s, 3H), 2.05-2.01 (3 s, 9H) + solvent peaks (heptane) ppm; HPLC (system A): t_R =5.1 min, purity = 91% (at 260 nm); LRMS (ESI+): *m/z* 603.9 (100) $[M + H]^+$, calcd for $C_{28}H_{30}NO_{14}^+$ 604.5; LRMS (ESI-): *m/z* 648.0 (100) $[M + H + FA]^-$, calcd for $C_{29}H_{30}O_{16}^-$ 648.5.

"Dual-analytes" probe β-Gal / NTR *(SD094)*

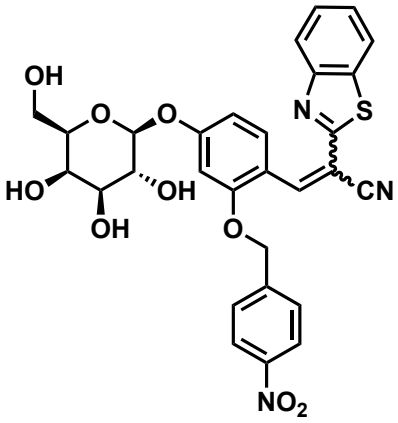


SD093 (173 g, 0.29 mmol, 1 equiv.) and malonitrile (20 mg, 0.30 mmol, 1.05 equiv.) were dissolved in absolute EtOH (15 mL). Then, 1 drop of piperidine was added and the resulting reaction mixture was stirred at RT for 1 h. Thereafter, volatiles were removed under vacuum and the resulting residue was dissolved in MeOH (27 mL). The mixture was cooled to 0 °C with an ice-water bath and a solution of MeONa (620 mg, 11.5 mmol, 40 equiv.) in MeOH (3 mL) was added and the reaction mixture was stirred for 2 h. The reaction was checked form completion by HPLC-MS (system A)[6]. Thereafter, the reaction mixture was quenched by adding glacial AcOH (1 mL), DCM (35 mL) was added and the whole mixture was filtered. The filtrate was concentrated, dissolved in EtOH (17 mL), then malonitrile (10 mg) was added and the mixture was

[6] To avoid formation of retro-Konevenagel side-product during the preparation of HPLC-MS samples, it is essential to quench the reaction mixture with AcOH instead of water.

stirred for further 1 h. Thereafter, the mixture was concentrated and directly purified by chromatography on a silica gel column (eluent: DCM-MeOH from 10 : 0 to 8 : 2, v/v). A impure product was recovered and a further purification by semi-preparative RP-HPLC was performed (system B, t_R = 56.3-58.9 min). The product-containing fractions were lyophilized to give the desired "dual-analytes" probe **SD094** as a white amorphous powder with yellow glow (15 mg, yield 8%).^{1}H NMR (300 MHz, DMSO-d_6): δ = 8.36 (s, 1H), 8.21 (d, *J* 8.7, 1H), 8.01 (d, *J* 8.7, 1H), 7.73 (d, *J* 8.7, 2H), 6.82 (s, 2H), 5.38 (s, 2H), 5.15 (d, *J* 5.1, 1H, O*H*), 4.90 (d, *J* 7.8, 1H), 4.83 (d, *J* 5.4, 1H, O*H*), 4.65 (t, *J* 5.1, 1H, O*H*), 4.48 (d, *J* 4.5, 1H, O*H*), 3.65-3.35 (m, 6H) ppm; IR (ATR): ν = 3386 (broad), 2935, 2239, 1605, 1578, 1527, 1502, 1459, 1435, 1386, 1349, 1322, 1273, 1216, 1193, 1144, 1101, 1072, 1030, 990, 938, 895, 840, 825, 787, 753, 735, 704 cm^{-1}; HPLC (system A): t_R = 4.3 min, purity = 97% (at 260 nm); LRMS (ESI +): *m/z* 484.2 (35) [M + H]$^+$, calcd for $C_{23}H_{22}N_3O_9^+$ 484.1; LRMS (ESI -): *m/z* 482.1 (45) [M - H]$^-$, calcd for $C_{23}H_{20}N_3O_9^-$ 482.1; UV-vis (PB, 25 °C): λ_{max} = 275 nm (ε 10 100 M^{-1}.cm^{-1}), 368 nm (ε 12 550 M^{-1}.cm^{-1}).

"Dual-analytes" probe β-Gal / NTR (mixture of two diastereomers) *(SD098)*



SD093 (252 mg, 0.67 mmol, 1 equiv.) and benzothiazole-2-acetonitrile (123 mg, 0.70 mmol, 1 equiv.) were dissolved in absolute EtOH (35 mL).Then, 1 drop of piperidine was added and the reaction mixture was stirred at RT for 1 h. Thereafter, volatiles were removed under vacuum and the resulting residue was dissolved in MeOH (67 mL). The mixture was cooled to 0 °C with an ice-water bath and a solution of MeONa (1.45 g, 26.9 mmol, 40 equiv.) in MeOH (8 mL) was added and the reaction mixture was stirred for 1 h. Thereafter, the reaction mixture was quenched by adding glacial AcOH (2 mL) and solvents were evaporated under reduced pressure. The resulting residue was crushed in a mixture of DCM-MeOH (7 : 3, v/v) to obtain a yellow glowing solid which was recovered by filtration, washed with deionized water and DCM. The product was slightly impure and was separated in two distinct batches for purification. The first batch (ca. 50 mg) was purified by semi-preparative RP-HPLC (system C, t_R = 8.9-12.7 min) to afford after lyophilization ca. 2 mg of desired product. The second batch was dissolved in a minimum amount of DMSO and the product was precipitated by adding MeOH and was collected by filtration to obtain 84 mg of **SD098** as yellow glowing solid (86 mg, yield 22%, mixture of two diastereomers). ^{1}H NMR (300 MHz, DMSO-d_6)[7]: δ = 8.62 (bs, 1H), 8.50-8.10 (m, 5H), 7.84 (bd, *J* 6.9, 2H), 7.59 (bdd, *J* 6.9 and 14.7, 2H), 6.98 (bs, 2H), 5.54 (bs, 2H), 5.28 (bs,1H), 5.03

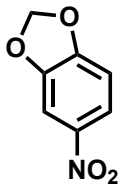
[7] All peaks are broad, certainly due to mixture of two diastereomers.

(bs, 1H), 4.96 (s, 1H), 4.79 (bs, 1H), 4.60 (bs, 1H), 3.78-3.50 (m, 6H) ppm; ^{13}C NMR (125 MHz, DMSO-d_6): major diastereomer, δ = 164.2, 162.9, 159.2, 153.5, 147.7, 144.7, 141.7, 134.5, 130.0, 128.6 (2C), 127.6, 126.6, 124.3 (2C), 123.5, 122.9,117.1, 115.4, 110.0, 103.0, 102.2, 100.9 (comb.)[8], 76.4, 73.8 (comb.), 70.5 (comb.), 69.5, 68.7 (comb.), 60.9 (comb.) ; minor isomer, δ = 161.9, 160.6, 157.7, 152.0, 147.5, 146.9, 144.4, 135.0, 132.4, 128.7 (2C), 127.3, 127.0, 124.0 (2C), 123.6, 122.8, 118.4, 115.5, 109.3, 108.5, 102.1, 100.9 (comb.), 76.3, 73.8 (comb.), 70.5 (comb.), 69.1, 68.7 (comb.), 60.9 (comb.) ppm; IR (ATR): ν = 3386 (broad), 2934, 2879, 2219 (weak), 1612, 1584, 1541, 1506, 1460, 1438, 1387, 1349, 1304, 1279, 1207, 1190, 1135, 1084, 1052, 1034, 1014, 947, 897, 880, 862, 841, 782, 758, 732, 725, 681 cm^{-1}; HPLC (system A): t_R = 4.7 min, purity = 98% (at 260 nm); LRMS (ESI+): *m/z* 591.9 (100) $[M + H]^+$, calcd for $C_{29}H_{25}N_3O_9S^+$ 592.5; LRMS (ESI -): *m/z* 590.1 (50) $[M - H]^-$, calcd for $C_{29}H_{23}N_3O_9S^-$ 590.5; UV-vis (PB, 25 °C): λ_{max} = 370 nm (ε 9 300 M^{-1}.cm^{-1}).

Please note: all fluorescence assays and HPLC-MS analyses related to *in-vitro* activation of fluorogenic probes **SD094** and **SD098** by β-Gal and NTR were performed as described in publication n°1, by replacing hydrolase (PEL or PGA) by β-Gal.

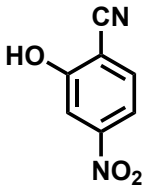
Highlighted Compounds of chapter 2

5-Nitro-1,3-benzodioxole[9] *(SD126E1, N° CAS : 2620-44-2, commercial chemical cmpd)*



1,3-Benzodioxole (15.0 g, 123 mmol, 1 equiv.) was added dropwise on 30 min to a solution of conc. HNO_3 (68 %, 28 mL, 430 mmol, 3.5 equiv.) in deionized H_2O (60 mL) at 65 °C. The mixture was then heated and stirred at 90 °C for 2 h. The mixture was cooled to RT and poured into ice / water (300 mL). The yellow solid formed, was recovered by filtration and finally dried to give **SD126E1** (21.0 g, quantitative yield). This compound was directly used in the next step. HPLC (system A): t_R = 3.9 min, purity = 99% (at 260 nm).

2-Hydroxy-4-nitrobenzonitrile[10] *(SD126E2, N° CAS : 39835-14-8 , commercial chemical cmpd)*



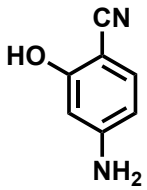
[8] Signals are combined for two diastereomers.

[9] X. Cai, C. Qian and H. Zhai, *WO2008115719 A1*, **2008**.

[10] Y. Imakura, K. Okimoto, T. Konishi, M. Hisazumi, J. Yamazaki, S. Kobayashi and S. Yamashita, *Chem. Pharm. Bull.*, **1992**, (40), 1691-1696.

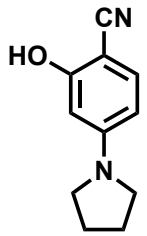
Under Ar atmosphere, **SD126E1** was added incrementally over 3 min to a solution of NaCN (18 g, 369 mmol, 3 equiv.) in HMPA (250 mL) at 150 °C. The resulting reaction mixture was stirred for 20 min and then quenched by sequential addition of ice / water (ca. 150 mL) and aq. 1.0 M NaOH (150 mL). The mixture was then extracted with Et_2O. The aqueous layer was acidified to pH 4-5 with aq. 10% HCl and extracted with Et_2O (3 × 150 mL). The combined organic phases were washed with brine (100 mL), dried over anhydrous Na_2SO_4, filtered and concentrated under vacuum. The resulting residue was purified by chromatography on a silica gel (eluent: heptane-EtOAc, step gradient from 10 : 0 to 7 : 3, v/v) to give one batch of pure benzonitrile derivative **SD126E2F1** (2.92 g, white solid) and one less pure fraction **SD126E2F2** (14.52 g, red solid) with a total yield of 82%. The combined fractions were used in the next step without further purification. HPLC (system A): t_R = 3.3 min, purity = 94 % (at 260 nm). LRMS (ESI-): *m/z* 163.3 (100) $[M - H]^-$, calcd for $C_7H_5N_2O_3^-$ 163.1.

4-Amino-2-hydroxybenzonitrile[11] *(SD126, N° CAS : 67608-58-6, commercial chemical cmpd)*



Under Ar atmosphere, nitro derivative **SD126E2** (15.714 g, 95.7 mmol, 1 equiv.) was dissolved in MeOH (1 L) and then Pd / C 5% (10,0 g, 0.05 equiv.) was added and the mixture was put under H_2 atmosphere for 2 h. The mixture was filtered on Dicalite® pad (to remove Pd / C) and concentrated under vacuum. The resulting residue was purified by chromatography on a silica gel column (eluent: heptane-EtOAc, step gradient from 7 : 3 to 1 : 9, v/v) to provide aniline **SD126** (8.19 g, yield 63%) as two distinct fractions **SD126F1** (brown solid, 6.91 g, purity = 95%) and **SD126F2** (yellow solid, 1.63 g, purity = 100%).^{1}H NMR (500 MHz, CD_3OD): δ = 7.14 (d, *J* 8.5, 1H), 6.17 (dd, *J* 2.0 and 8.5, 1H), 6.13 (d, *J* 2.0, 1H) ppm; HPLC (system A): t_R = 1.7 min, purity = 98% (at 260 nm); LRMS (ESI+): *m/z* 135.3 (100) $[M + H]^+$, calcd for $C_7H_7N_2O^+$ 135.1; LRMS (ESI-): *m/z* 133.4 (100) $[M - H]^-$, calcd for $C_7H_7N_2O^-$ 133.1. *Overall yield for this 3-step procedure : 52%*

2-Hydroxy-4-(1-pyrrolidinyl)benzonitrile *(SD136, N° CAS : 1784519-69-2, commercial chemical cmpd)*

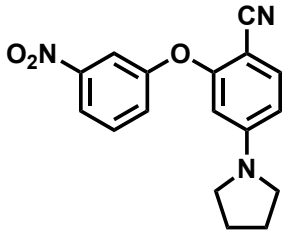


Aniline **SD126** (250 mg, 1.86 mmol, 1 equiv.) was dissolved in toluene (2.3 mL) and 1,4 dibromobutane (222 μL, 1.86 mmol, 1 equiv.) and DIEA (800 μL, 4.66 mmol, 1

[11] S. Noël, F. Hoegy, F. Rivault, D. Rognan, I. J. Schalk and G. L. A. Mislin, *Bioorg. Med. Chem. Lett.*, **2014**, (24), 132-135.

equiv.) were sequentially added. The resulting reaction mixture was stirred at 120 °C (reflux) overnight. Thereafter, the reaction was quenched by adding glacial AcOH (0.3 mL). The mixture was extracted with EtOAc and washed with brine, dried over anhydrous $MgSO_4$, filtered and concentrated under vacuum. The resulting residue was purified by chromatography on a silica gel column (eluent: heptane-EtOAc, step gradient from 8 : 2 to 5 : 5, v/v) to afford the pyrrolidine **SD136** as yellow solid (48 mg, yield 13%). ^{1}H NMR (500 MHz, DMSO-d_6): 10.44 (s,1H, O*H*), 7.26 (d, *J* 8.5, 1H), 6.10 (dd, *J* 2.5 and 8.5, 1H), 6.01 (d, *J* 2.0, 1H), 3.23 (t, *J* 6.5, 4H), 1.94 (bquint., *J* 4.0, 4H) ppm; HPLC (system A): t_R = 4.13 min, purity = 98% (at 260 nm); LRMS (ESI+): *m/z* 189.3 (70) $[M + H]^+$, calcd for $C_{11}H_{13}N_2O^+$ 189.2; LRMS (ESI-): *m/z* 187.5 (100) $[M - H]^-$, calcd for $C_{11}H_{13}N_2O^-$ 187.2.

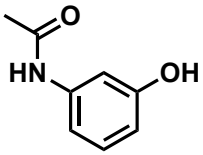
Mixed bis-aryl ether *(SD137)*



Phenol **SD136** (100 mg, 0.53 mmol, 1 equiv.) and anhydrous K_2CO_3 (81 mg, 0.58 mmol, 1.1 equiv.) were dissolved in dry DMF (1 mL) and the mixture was stirred at 150 °C for 5 min. Then, 1-fluoro-3-nitrobenzene (83 mg, 0.58 mmol, 1.1 equiv.) was added and the reaction mixture was stirred at 150 °C overnight. Thereafter, the mixture was put in ice / water and extracted with EtOAc. The organic layer was dried over anhydrous $MgSO_4$, filtered and concentrated under vacuum. The resulting residue was purified by chromatography on a silica gel column (eluent: heptane-EtOAc, step gradient from 7 : 3 to 6 : 4, v/v) to provide the mixed bis-aryl ether **SD137** as a yellow solid (24 mg, yield 14%). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.98 (dd, *J* 8.0 and 2.0, 1H), 7.81 (t, *J* 2.5, 1H), 7.53 (t, *J* 8.5, 1H), 7.46 (d, *J* 9.0, 1H), 7.40 (dd, *J* 2.5 and 8.0, 1H), 6.37 (dd, *J* 2.5 and 9.0, 1H), 6.06 (d, *J* 2.5, 1H), 3.26 (t, *J* 7.0, 4H), 2.02 (bquint., *J* 7.0, 4H); HPLC (system A): t_R = 5.4 min, purity = 96% (at 260 nm); LRMS (ESI+): *m/z* 310.3 (25) $[M + H]^+$, calcd for $C_{17}H_{13}N_3O_3^+$ 310.3 and 351.4 (100) $[M + H + CH_3CN]^+$, calcd for $C_{19}H_{19}N_3O_3^+$ 310.3

Aldehydic precursor of "triple-gated & dual-analytes" probe NTR / PGA (4-step procedure)

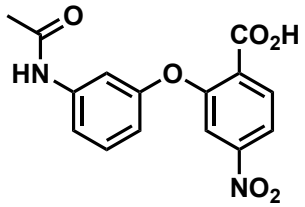
3-Acetamidophenol[12] *(SD201E1, N° CAS : 621-42-1, commercial chemical cmpd)*



[12] For experimental NMR and MS spectra, see : a) S. S. van Berkel, B. van der Lee, F. L. van Delft and F. P. J. T. Rutjes, *Chem. Commun.*, **2009**, 4272-4274 ; b) J. R. Heys et C. S. Elmore, *J. Labelled Compd. Radiopharm.*, **2009**, (52), 189-200.

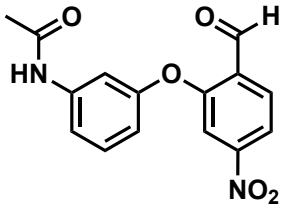
Ac_2O (865 μL, 9.1 mmol, 1 equiv.) was added to a solution of *meta*-aminophenol (1.0 g, 9.10 mmol, 1 equiv.) in dry THF (40 mL) and stirred at RT for 2 h. Thereafter, the crude mixture was concentrated and triturated in toluene to afford acetamide **SD201E1** as white solid (1.17 g, yield 83%). HPLC (system A): t_R = 3.1 min, purity = 100% (at 260 nm); LRMS (ESI+): *m/z* 152.5 (100) $[M + H]^+$, calcd for $C_8H_9NO_2^+$ 152.1; LRMS (ESI-): *m/z* 150.4 (100) $[M - H]^-$, calcd for $C_8H_8NO_2$ 150.1.

2-[3-(Acetylamino)phenoxy]-4-nitrobenzoic acid[13] *(SD201E2, N° CAS : 875843-72-4)*



Phenol **SD201E1** (1.15 g, 7.60 mmol, 1.1 equiv.), 4-nitro-2-chlorobenzoic acid (1.39 g, 6.9 mmol, 1 equiv.), anhydrous K_2CO_3 (1.05 g, 7.60 mmol, 1.1 equiv.) and Cu (66 mg, 1.03 mmol, 0.15 equiv.) were mixed (suspension at RT) in dry DMF (20 mL) and stirred at 130 °C overnight. Thereafter, the reaction mixture was put in a mixture of aq. 1.0 M HCl 1N and ice. The formed precipitate was filtered and washed with Et_2O. The recovered solid was sonicated in MeOH and the resulting suspension was filtered. MeOH phase was concentrated under vacuum to give **SD201E2** as dark brown solid (770 mg, yield 36%). ^1H NMR (300 MHz, DMSO-d_6): δ = 10.06 (s, 1H), 8.03 (m, 2H), 7.65 (d, *J* 1.8, 1H), 7.41-7.33 (m, 3H), 6.74 (m, 1H), 2.02 (s, 3H) ppm; HPLC (system A): t_R = 4.1 min, purity = 91% (at 260 nm); LRMS (ESI+): *m/z* 317.3 (40) $[M + H]^+$, calcd for $C_{15}H_{12}N_2O_6^+$ 317.3; LRMS (ESI-): *m/z* 315.2 (70) $[M - H]^-$, calcd for $C_{15}H_{10}N_2O_6^-$ 315.3.

2-[3-(Acetylamino)phenoxy]-4-nitrobenzaldehyde[14] *(SD201)*



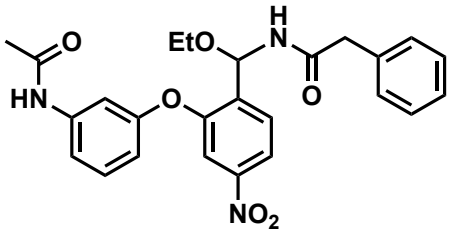
Benzoic acid **SD201E2** (750 mg, 2.37 mmol, 1 equiv.), *N*,*O*-dimethylhydroxylamine hydrochloride (255 mg, 2.61 mmol, 1.1 equiv.) and carbodiimide EDCI (546 mg, 2.85 mmol, 1.2 equiv.) were dissolved in dry DCM (3 mL). TEA (826 μL, 5.93 mmol, 2.5 equiv.) was added dropwise and the reaction mixture was stirred at RT for 3 days. Thereafter, the reaction mixture was quenched with aq. sat. $NaHCO_3$, diluted with deionized water and extracted with DCM (the black suspension is also put in the organic layer). The organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated under vacuum. The Weinreb amide was directly dissolved in dry DCM (5 mL) under Ar athmosphere and cooled to -82 °C with a

[13] J. Cui, J. Jin, Y.-H. Hsieh, H. Yang, B. Ke, K. Damera, P. C. Tai and B. Wang, *ChemMedChem*, **2013**, (8), 1384-1393.

[14] Adapted from : K. A. Leonard, K. Barbay, J. P. Edwards, K. D. Kreutter, D. A. Kummer, U. Maharoof, R. Nishimura, M. Urbanski, H. Venkatesan, A. Wang, R. L. Wolin, C. R. Woods, J. Pierce, S. Goldberg, A. Fourie and X. Xue, *US20140107094 A1*, **2014**.

EtOAc-liquid N_2 bath. Then, DIBAL-H (1.0 M in toluene, 5.45 mL, 2.3 equiv.) was added dropwise over 40 min. The resulting mixture was stirred at -82 °C for further 10 min before to be quenched by adding MeOH and aq. sat. NH_4Cl (50 mL). After 30 min of stirring, the mixture was filtered on a Dicalite® pad and DCM was added before phase separation. The organic layer was washed with aq. 1.0 M HCl, dried over anhydrous $MgSO_4$, filtered and concentrated under vacuum. Purification by chromatography on a silica gel column (eluent: DCM-EtOAc, a step gradient from 10 : 0 to 7 : 3, v/v) provided benzaldehyde **SD201** as yellow / orange solid (can be an oil too) (315 mg, yield 44%, overall yield 13% for 4 steps). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 10.57 (s, 1H), 8.06 (d, *J* 8.7, 1H), 7.94 (dd, *J* 2.1 and 8.7, 1H), 7.67 (d, *J* 2.1, 3H), 7.37 (t, *J* 8.1, 1H), 7.20 (dd, *J* 1.5 and 7.5, 1H), 6.85 (dd, *J* 2.1 and 7.8, 1H), 2.17 (s, 3H) + solvent peak (EtOAc) ppm; HPLC (system A): t_R = 4.3 min, purity = 100% (at 260 nm); LRMS (ESI+): *m/z* 342.0 (10) $[M + H + CH_3CN]^+$, calcd for $C_{17}H_{16}N_3O_5^+$ 342.3; LRMS (ESI-): *m/z* 299.2 (50) $[M - H]^-$, calcd for $C_{15}H_{11}N_2O_5^-$ 299.3.

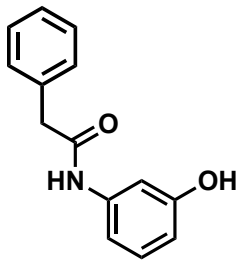
"Triple-gated & dual-analytes" probe NTR / PGA[15] *(SD203)*



Under Ar atmosphere, benzaldehyde **SD201** (150 mg, 0.50 mmol, 1 equiv.) and phenylacetamide (81 mg, 0.60 mmol, 3 equiv.) were dissolved in dry DCM (1.7 mL). A solution of $Ti(OEt)_4$ (white gelatinous residue at RT in commercial bottle, purity not determined, ca. 170 mg, 0.75 mmol, 1.5 equiv.) in dry DCM (0.3 mL) was added dropwise (the suspended white solid was not injected in the reaction mixture) to the previous solution and the resulting reaction mixtrue was stirred at RT for 8 h. Then, EtOH (0.5 mL) was added to the mixture and the solution was stirred overnight. Thereafter, a further amount of $Ti(OEt)_4$ (100 mg in 0.3 mL of dry DCM) was added to the mixture which was stirred for further 3 days (monitored HPLC-MS, system A). Finally, the reaction mixture was concentrated under vacuum and the resulting residue was dissolved in a mixture of CH_3CN-H_2O (4 : 3, v/v) and purified by automated flash-column chromatography over reversed-phase C_{18} silica gel (RP-C_{18} cartridge, SiliCycle siliaSep™ C_{18}, 40 g) using a linear gradient of CH_3CN in H_2O (from 50% to 100%, 15 column vol.). The product-containing fractions were lyophilized to give the probe **SD203** as white-off solid (25 mg, yield 11%). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 10.0 (s, 1H, N*H*), 8.87 (d, *J* 9.3, 1H), 8.00 (dd, *J* 2.1 and 8.4, 1H), 7.84 (d, *J* 8.7, 1H), 7.45 (d, *J* 2.1, 1H), 7.39 (s, 1H, N*H*), 7.28-7.11 (m, 7H), 6.59 (m, 1H), 6.33 (d, *J* 9.0, 1H), 3.56-3.48 (m, 4H), 1.97 (s, 3H), 1.04 (t, *J* 7.2, 3H) ppm; IR (ATR): ν = 3403, 1682, 1658, 1603, 1522, 1481, 1440, 1412, 1345, 1308, 1281, 1238, 1198, 1139, 1124, 1096, 1079, 1063, 1031, 1014, 973, 895, 877, 849, 819, 793, 764, 733, 698 cm^{-1}; HPLC (system A): t_R = 4.7 min, purity = 95% (at 260 nm); LRMS (ESI-): *m/z* 462.2 (40) $[M - H]^-$, calcd for $C_{25}H_{24}N_3O_6^-$ 462.5 and 508.1 (100) $[M + HCOO]^-$, calcd for $C_{26}H_{26}N_3O_8^-$ 508.5.

[15] M. Li, B. Luo, Q. Liu, Y. Hu, A. Ganesan, P. Huang and S. Wen, *Org. Lett.*, **2014**, (16), 10-13.

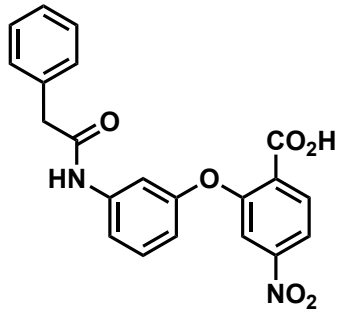
N-(3-Hydroxyphenyl)benzeneacetamide[16] (*SD213, N° CAS : 23478-26-4, commercial chemical cmpd)*



meta-Aminophenol (2.0 g, 18.3 mmol, 1 equiv.) was dissolved in dry THF (60 mL) and pyridine (1.63 mL, 20.15 mmol, 1.1 equiv.) was added; the mixture was cooled to 0 °C. Phenylacetyl chloride (PhAcCl, 2.45 mL, 18.5 mmol, 1.01 equiv.) was added dropwise to the mixture which was then allowed to reach RT and was stirred for 4 h 30. Thereafter, the mixture was concentrated and retaken with EtOAc. The organic layer was washed with aq. sat. NH_4Cl and brine, dried over anhydrous $MgSO_4$, filtered and concentrated under vacuum. The resulting residue was purified by chromatography on a silica gel column (eluent: DCM-EtOAc, a step gradient from 10 : 0 to 5 : 5, v/v) to give phenylacetamide **SD213** as grey solid (2.60 g, yield 62%). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 10.0 (s, 1H), 9.34 (s, 1H), 7.32 (d, *J* 4.5, 4H), 7.32 (m, 1H), 7.17 (t, *J* 2.1, 1H), 7.06 (t, *J* 8.1, 1H), 6.95 (dt, *J* 1.8 and 8.1, 1H), 6.44 (ddd, *J* 0.9, 2.4 and 7.8, 1H), 3.61 (s, 2H) ppm; HPLC (system A): t_R = 3.7 min, purity = 99% (at 260 nm); LRMS (ESI+): *m/z* 228.1 (100) $[M + H]^+$, calcd for $C_{14}H_{14}NO_2^+$ 228.1; LRMS (ESI-): *m/z* 226.1 (100) $[M - H]^-$, calcd for $C_{14}H_{12}NO_2^-$ 226.1.

Precursor of NTR probe "PABA-like" (2-step procedure)

2-[3-(Phenylacetamino)phenoxy]-4-nitrobenzoic acid[13] (*SD222)*

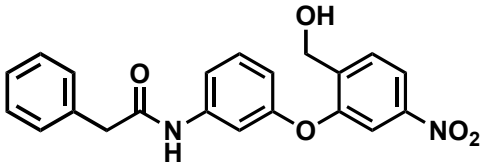


Phenol **SD213** (1.20 g, 5.28 mmol, 1.1 equiv.), 4-nitro-2-chlorobenzoic acid (968 mg, 4.80 mmol, 1 equiv.), anhydrous K_2CO_3 (730 mg, 5.28 mmol, 1.1 equiv.) and Cu (50 mg, 0.80 mmol, 0.15 equiv.) were mixed (suspension at RT) in dry DMF (13 mL) and the mixtrue was stirred at 130 °C overnight. Thereafter, the reaction mixture was put in a mixtrue of aq. 1.0 M HCl and ice. The formed precipitate was filtered and the

[16] For experimental NMR spectra, see : A. Sun, A. Prussia, W. Zhan, E. E. Murray, J. Doyle, L.-T. Cheng, J.-J. Yoon, E. V. Radchenko, V. A. Palyulin, R. W. Compans, D. C. Liotta, R. K. Plemper and J. P. Snyder, *J. Med. Chem.*, **2006**, (49), 5080-5092.

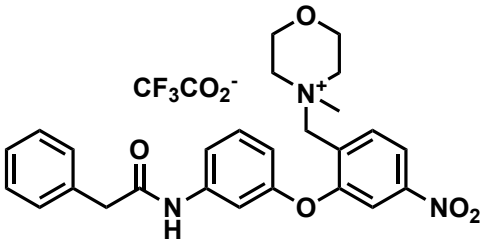
recovered solid was sonicated in Et_2O. The resulting solution was filtered and the organic phase was concentrated under vacuum to give mixed bis-aryl ether **SD222** as an impure dark brown solid which was directly used in the next step. HPLC (system A): t_R = 4.6 min, purity = 29% (at 260 nm). LRMS (ESI+): *m/z* 393.1 (100) [M + H]$^+$, calcd for $C_{21}H_{17}N_2O_6^+$ 393.4; LRMS (ESI-): *m/z* 391.1 (100) [M - H]$^-$, calcd for $C_{21}H_{15}N_2O_6^-$ 391.1.

2-[3-(Phenylacetamino)phenoxy]-4-nitrobenzyl alcohol[13] (SD225)



A mixture of benzoic acid **SD222** (5.28 mmol assumed) and Castro's reagent (BOP, 2.34 g, 5.28 mmol, 1.0 equiv.) was suspended in dry THF (25 mL). DIEA (1 mL, 5.76 mmol, 1.2 equiv.) and $NaBH_4$ (181 mg, 4.80 mmol, 1 equiv.) were sequentially added (10 min break between the two additions).The mixture was stirred at RT for 40 min. Thereafter, the reaction mixture was concentrated under vacuum and was retaken with Et_2O and filtered to remove the precipitate (boron salts). The organic layer was washed with aq. 5% HCl and aq. sat. $NaHCO_3$, dried over anhydrous $MgSO_4$, filtered and concentrated under vacuum. The resulting residue was purified by chromatography on a silica gel column (dry loading, eluent: heptane-EtOAc, a step gradient from 9 : 1 to 5 : 5, v/v) to afford benzylic alcohol **SD225** as yellow oil (102 mg, overall yield 6% for 2 steps). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.82 (dd , *J* 2.1 and 8.4, 1H), 7.56 (s + d, 2H), 7.46 (d, *J* 2.1, 1H), 7.33-7.12 (m, 7H + $CHCl_3$), 6.92 (dd, *J* 2.1 and 8.1, 1H), 6.63 (dd, *J* 1.5 and 8.1, 1H), 4.69 (s, 2H), 3.58 (s, 2H), 2.80 (bs, 1H, O*H*) ppm; HPLC (system A): t_R = 4.7 min, purity = 100% (at 260 nm); LRMS (ESI+): *m/z* 379.1 (100) [M + H]$^+$, calcd for $C_{21}H_{19}N_2O_3^+$ 379.1; LRMS (ESI-): *m/z* 377.1 (100) [M - H]$^-$, calcd for $C_{21}H_{19}N_2O_3$ 377.1.

Water-soluble NTR probe ("PABA-like" strategy) *(SD242)*

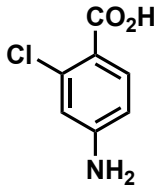


A solution of benzylic alcohol **SD225** (90 mg, 0.24 mmol, 1 equiv.) and DIEA (45 μL, 0.26 mmol, 1.1 equiv.) in dry toluene (0.2 mL) was added dropwise to a solution of phosgene (15% in toluene, 187 μL, 0.26 mmol, 1.1 equiv.) pre-cooled at 0 °C. The mixture was stirred for 2 h and then concentrated under vacuum. The residue was dissolved in dry NMP (2 mL) and added dropwise to a solution of tributylammonium salt of 2-aminoethane-1,1-disulfonic acid[17] in NMP (0.3 M, 4 mL, 1.2 mmol, 5 equiv.) pre-cooled at 0 °C. The reaction mixture was stirred 30 min. The reaction was

[17] a) A. Romieu, D. Tavernier-Lohr, S. Pellet-Rostaing, M. Lemaire and P.-Y. Renard, *Tetrahedron Lett.*, **2010**, (51), 3304-3308 ; b) A. Romieu, C. Massif, S. Rihn, G. Ulrich, R. Ziessel and P.-Y. Renard, *New J. Chem.*, **2013**, (37), 1016-1027.

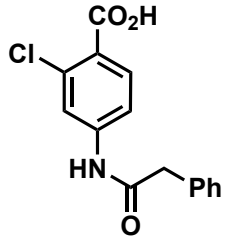
checked for completion by HPLC-MS (system A). Benzyl chloride derivative **SD242A** was identified as the major product (t_R = 5.3 min, ESI+: *m/z* 397.6 (30) & 399.4 (10) $[M + H]^+$; , ESI -: *m/z* 395.5 (100) & 397.5 (40) $[M - H]^-$). Thus, NMM (560 µL, 20 equiv.) was added to the reaction mixture and heated to 110 °C for 30 min. Thereafter, the excess of NMM was evaporated and the mixture was disslved with ultrapure water (7 mL) and purified by semi-preparative HPLC (system D, t_R = 25.9-30.3)[18]. The product-containing fractions were lyophilized to give the TFA salt of probe **SD242** as a light brown solid (21 mg, yield 20%). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 10.43 (s, 1H), 8.09 (dd, *J* 2.1 and 8.4, 1H), 7.94 (d, *J* 8.4, 1H), 7.71 (t, *J* 2.1, 1H), 7.50 (d, *J* 2.1, 1H), 7.45 (d, *J* 8.1, 1H), 7.39-7.21 (m, 6H), 6.94 (ddd, *J* 1.2, 2.1 and 7.8, 1H), 4.89 (s, 2H), 4.00 (bs, 4H), 3.65 (bs, 4H), 3.18 (s, 3H), 2.09 (s, 2H) ppm; ^{19}F NMR (282 MHz, DMSO-d_6): δ = -73.9 (s, 3F, CF_3-TFA) ppm; IR (ATR): ν = 1670 (broad), 1600, 1525, 1484, 1419, 1348, 1279, 1235, 1189, 1122, 1061, 1018, 983, 956, 876, 818, 797, 717, 695 cm^{-1}; HPLC (system A): t_R = 3.9 min, purity = 97% (at 260 nm); LRMS (ESI+): *m/z* 462.7 (100) $[M]^{+\circ}$, calcd for $C_{26}H_{28}N_3O_5^+$ 462.5; LRMS (ESI-): *m/z* 460.5 (10) $[M^{+\circ} - 2H]^-$, calcd for $C_{26}H_{26}N_2O_5^-$ 460.5; Elemental anal.: Found C, 54.4; H, 5.1; N, 6.6. $C_{26}H_{28}N_3O_5^+$.1.0 $CF_3CO_2^-$.0.5 CF_3CO_2H requires C, 55.1; H, 4.54; N, 6.6; UV (PB, 25 °C): λ_{max} = 230 nm (ε 21 640 $M^{-1}.cm^{-1}$), 319 nm (ε 2 710 $M^{-1}.cm^{-1}$).

4-Amino-2-chlorobenzoic acid[19] *(SD239, N° $_{CAS}$: 2457-76-3, commercial chemical cmpd)*



4-Nitro-2-chlorobenzoic acid (10 g, 49.6 mmol, 1 equiv.) was dissolved in MeOH (100 mL) under Ar atmosphere, then Pd / C 5% (5.8 g, 0.05 equiv.) was added and the mixture was put under H_2 atmosphere for 24 h. The reaction mixture was dissolved in DCM, filtered on a Dicalite® pad (to remove Pd / C) and concentrated to obtain aniline **SD239** as yellow-brown solid (8.38 g, yield 98%).^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.64 (d, *J* 8.7, 1H), 6.61 (d, *J* 2.1, 1H), 6.50 (dd, *J* 8.7 and 2.1, 1H) (NH_2 and CO_2H are not visible (under the water's peak for NH_2 ?)) ppm.

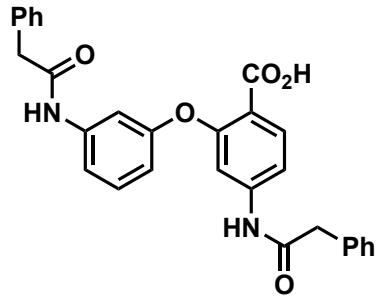
2-Chloro-4-(2-phenylacetamido)benzoic acid[20] *(SD244, N° $_{CAS}$: 1030676-56-2, commercial chemical cmpd)*



[18] Due to the large volume, several injections (2 mL) will be done under initial conditions before starting the elution gradient ("step-by-step injection" or " incremental injection").
[19] A. Gangjee, H. D. Jain, J. J. McGuire and R. L. Kisliuk, *J. Med. Chem.*, **2004**, (47), 6730-6739.
[20] Adapted from : A. J. Harte and T. Gunnlaugsson, *Tetrahedron Lett.*, **2006**, (47), 6321-6324.

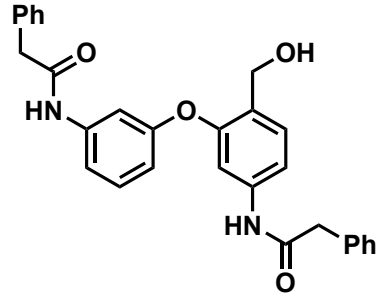
Aniline **SD239** (1.0 g, 5.82 mmol,1 equiv.) and NaOH pellets (466 mg, 11.64 mmol, 2 equiv.) were dissolved in deionized water (80 mL) and the resulting solution was cooled to 0 °C. Then, PhAcCl (2.3 mL, 17.46 mmol, 3 equiv.) was added dropwise to the mixture which was then allowed to reach RT and was stirred overnight. Thereafter, the mixture was put in a mixture of aq. 1.0 M HCl and ice. The formed precipitate was filtered and sonicated in Et_2O and filtered again. PE was added to the filtrate to precipitate the desired product. **SD244** was recovered by filtration and dried at 80 °C overnight to give a white cream solid (855 mg, yield 51%). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 13.10 (bs, 1H, $CO_2\underline{H}$), 10.58 (s, 1H, N$\underline{H}$), 7.89 (s,1H), 7.83 (d, *J* 8.7, 1H), 7.55 (d, *J* 8.1, 1H), 7.33-7.25 (m, 5H), 3.68 (s, 2H) ppm.

Mixed bis-aryl ether *(SD247)*



Aryl chloride **SD244** (400 mg, 1.38 mmol, 1 equiv.), phenol **SD213** (344 mg, 1.52 mmol, 1.1 equiv.), anhydrous K_2CO_3 (210 mg, 1.52 mmol, 1.1 equiv.) and Cu (14 mg, 0.21 mmol, 0.15 equiv.) were mixed (suspension at RT) in dry DMF (13 mL) and the reaction mixture was stirred at 130 °C overnight. Thereafter, the reaction mixture was put in a mixtrue of aq. 1.0 M HCl and ice. The formed precipitate was recovered by filtration. This solid was dissolved in a mixture of Et_2O and EtOAc and the resulting solution was filtered again. The filtrate was concentrated under vacuum to give the impure mixed bis-aryl ether **SD247** which was directly used in the next step without further purification.

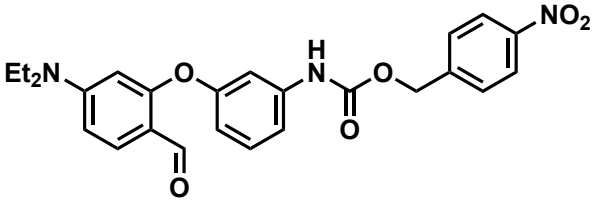
Benzyl alcohol *(SD249)*



Benzoic acid **SD247** and Castro's reagent (BOP, 671 mg, 1.52 mmol, 1.1 equiv.) were suspended in dry THF (7 mL) and DIEA (288 μL, 1.66 mmol, 1.2 equiv.) and $NaBH_4$ (52 mg, 1.38 mmol, 1 equiv.) were sequentially added (10 min break between the two additions).The mixture was stirred at RT for 1 h. Thereafter, the reaction mixture was concentrated under vacuum. The resulting residue was dissolved in EtOAc, washed successivley with aq. 5% HCl, aq. sat. $NaHCO_3$, brine, dried over anhydrous $MgSO_4$, filtered and concentrated under vacuum. The residue was

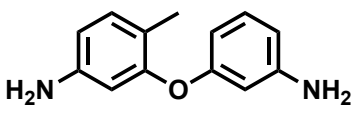
purified by chromatography on a silica gel column (eluent: heptane-EtOAc, a step gradient from 9 : 1 to 5 : 5, v/v) to provide the desired benzyl alcohol **SD249** as an impure yellow oil (100 mg, overall yield 15% for 2 steps). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 10.60 (s, 1H), 10.35 (s,1H), 8.05 (d, *J* 8.7, 1H), 7.81 (d, *J* 8.7, 2H), 7.61 (s, 1H), 7.40-7.24 (m, 12H), 6.94 (dt, *J* 1.5 and 7.8, 1H), 3.71 (s, 2H), 3.65 (s, 2H), 3.60 (s, 2H) ppm.

NTR probe[22] *(SD316)*



In a sealed tube, 4-(*N*,*N*-diethylamino)salicylaldehyde[21] (N° $_{CAS}$: 17754-90-4, 200 mg, 1.03 mmol, 1.2 equiv.), CuI (17 mg, 0.09 mmol, 0.1 equiv.), K_3PO_4 (367 mg, 1.72 mmol, 2 equiv.) and picolinic acid (21 mg, 0.17 mmol, 0.2 equiv.) were introduced under Ar atmosphere. Thereafter, DMSO (2 mL) and *meta*-iodoaniline (189 mg, 0.86 mmol, 1 equiv.) were added and the mixture was stirred at 85 °C overnight. After cooling to RT, 4-nitrobenzyl chloroformate (223 mg, 1.03 mmol, 1.2 equiv.) was introduced directly and slowly to the mixture and was stirred at RT further 30 min. Thereafter, a mixture of DCM and deionized water (10 : 1, v/v) was added to the crude, which was finally extracted with a further amount of DCM. The organic layer was washed with aq. 1.0 M HCl, brine and dried over anhydrous Na_2SO_4. After a rapid filtration on a plug of silica gel with EtOAc as eluent, the desired product was recovered (119 mg, yield 30%). A further purification by semi-preparative RP-HPLC (system E, t_R = 30.9-33.3 min) was required to get a high degree of purity compatible with fluorescence assays. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 10.13 (s, 1H, C*H*O), 8.27 (d, *J* 8.0, 2H), 7.86 (d, *J* 9.0, 1H), 7.59 (d, *J* 8.5, 2H), 7.31-7.19 (m, 3H), 6.97(s, 1H), 6.79 (d, *J* 8.0, 1H), 6.56 (d, *J* 8.5, 1H), 6.16 (s,1H), 5.33 (s, 2H), 3.39 (quad, *J* 7.5, 4H), 1.20 (t, *J* 7.0, 6H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 187.1, 161.4, 158.2, 153.0, 152.9, 148.0, 143.5, 139.3, 130.7, 130.6, 128.6 (2C), 124.1 (2C), 114.0, 113.7, 109.3, 108.6, 102.0, 65.7, 45.8 (2C), 12.5 (2C) ppm; IR (ATR): ν = 3255 (weak), 3198 (weak), 3082 (weak), 3046 (weak), 2975 (weak), 2932 (weak), 2857 (weak), 1732, 1643, 1592, 1548, 1513, 1482, 1456, 1444, 1406, 1392, 1378, 1344, 1300, 1267, 1223, 1206, 1173, 1150, 1103, 1073, 1013, 998, 980, 957, 872, 861, 843, 820, 799, 774, 760, 734, 705, 683 cm^{-1}; HPLC (system A): t_R = 5.4 min, purity = 97% (at 260 nm); LRMS (ESI+): *m/z* 464.2 (100) $[M + H]^+$, calcd for $C_{25}H_{26}N_3O_6^+$ 464.4. LRMS (ESI-): *m/z* 461.9 (100) $[M - H]^-$, calcd for $C_{25}H_{24}N_3O_6^-$ 462.5.

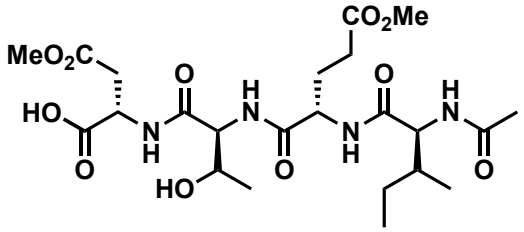
Mixed bis-aryl ether[22] *(SD336)*



[21] For its synthesis, see: S. Debieu and A. Romieu, *Org. Biomol. Chem.*, **2017**, (15), 2575-2584. This is also a commercial chemical cmpd.
[22] Adapted from : D. Maiti and S. L. Buchwald, *J. Am. Chem. Soc.*, **2009**, (131), 17423-17429.

In a sealed tube, 5-amino-*ortho*-cresol (809 mg, 6.57 mmol, 1.2 equiv.), CuI (52 mg, 0.27 mmol, 0.05 equiv.), K_3PO_4 (2.33 g, 10.96 mmol, 2 equiv.) and picolinic acid (68 mg, 0.55 mmol, 0.1 equiv.) were introduced under Ar atmosphere. Thereafter, DMSO (11 mL) and *meta*-iodoaniline (1.2 g, 5.48 mmol, 1 equiv.) were added and the mixture was stirred at 80 °C overnight. Thereafter, a mixtrue of EtOAc and deionized water (10 : 1, v/v) was added to the crude, which was finally extracted with a further amount of EtOAc. The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated under vacuum. A short purification by chromatography on a silica gel, (eluent: heptane-EtOAc 1 : 3, v/v) provided the bis-aniline **SD336** as brown oil (1.13 g, yield 96%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.03 (t, *J* 9.0, 1H), 6.97 (d, *J* 8.1, 1H), 6.39 (dd, *J* 2.4 and 7.8, 1H), 6.35-6.26 (m, 3H), 6.21 (t, *J* 2.1, 1H), 3.56 (bs, 4H, 2 × NH_2), 2.08 (s, 3H) ppm; HPLC (system A): t_R = 0.9 min, purity = 98% (at 260 nm); LRMS (ESI +): *m/z* 215.2 (100) $[M + H]^+$, calcd for $C_{13}H_{15}N_2O^+$ 215.3.

Tetrapeptide Ac-Ile-Glu(OMe)-Thr-Asp(OMe)-OH ***(SD334)***

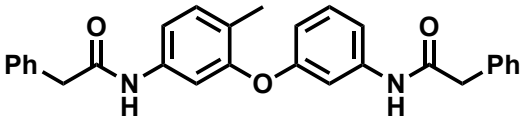


Tetrapeptide **Ac-Ile-Glu(OMe)-Thr-Asp(OMe)-OH** was obtained by automated solid-phase peptide synthesis on a Liberty Blue microwave synthesizer (CEM). The synthesis (0.25 mmol scale) was carried out on a Wang resin (0.70 mmol/g, 714 mg) using pre-defined CEM High Swelling cycles and standard Fmoc chemistry (DIC/ DMAP(2 : 0.1)) except for the Fmoc removal step (HOBt (C=0.1M) in 20% piperidine/DMF). Fmoc-L-Ile-OH and Fmoc-Thr(OtBu)-OH were purchased from Iris Biotech GmbH. Fmoc-L-Asp(OMe)-OH[23] (N° $_{CAS}$: 145038-53-5)[24] and Fmoc-L-Glu(OMe)-OH (N° $_{CAS}$: 145038-50-2) were prepared from H-L-Asp(OMe)-OH and H-L-Glu(OMe)-OH (obtained from Bachem) respectively and under standard conditions[25]. Acetylation of *N*-terminal side of the isoleucine was achieved by treatment with Ac_2O / pyridine (2.36 mL and 2.0 mL) in DMF (5 mL) at RT for 1 h. The peptide was cleaved from the resin with the simultaneous removal of tBu side-chain protecting group (Thr residue) with TFA / TIPS / H_2O (92.5 : 2.5 : 2.5 : 2.5, v/v, 10 mL) at RT for 2 h. The filtrate from the cleavage mixture was concentrated, precipitated in cold Et_2O, collected by centrifugation (twice), and lyophilized to afford crude peptide. This crude peptide was purified by semi-preparative RP-HPLC (system F, t_R = 33.0 to 35.1) and the pure cmpd **SD334** was obtained as white amorphous powder (50 mg, yield 18%); ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.11 (2 d, *J* 7.8, 2H), 7.94 (d, *J* 8.7, 1H), 7.67 (d, *J* 8.4, 1H), 4.59 (quad., *J* 7.8, 1H), 4.33 (quad., *J* 5.7, 1H), 4.21-4.11 (m, 2H), 3.93 (m, 1H), 3.59 (d, *J* 3.9, 1H), 2.82-2.65 (m, 2H), 2.36-0.79 (m, 18H) ppm. Two protons are not visible (OH, bs at 4.82 ? and CO_2H); HPLC (system A): t_R = 3.1 min, purity = not determined; LRMS (ESI+): *m/z*

[23] A. G. Jamieson, N. Boutard, K. Beauregard, M. S. Bodas, H. Ong, C. Quiniou, S. Chemtob and W. D. Lubell, *J. Am. Chem. Soc.*, **2009**, (131), 7917-7927.
[24] These two Fmoc-L-amino acids are commercially available but in our case, they were synthesized "in house" by Mr. Ludovic Palladino (bachelor of chemistry, University of Burgundy, training period January 2017).
[25] L. Lapatsanis, G. Milias, K. Froussios and M. Kolovos, *Synthesis*, **1983**, 671-673.

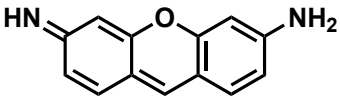
547.1 (100) $[M + H]^+$, calcd for $C_{23}H_{39}N_4O_{11}^+$ 547.6; LRMS (ESI-): *m/z* 545.0 (100) $[M - H]^-$, calcd for $C_{23}H_{37}N_4O_{11}^-$ 545.6.

Mixed bis-aryl ether[26] *(SD341)*



Bis-aniline **SD336** (200 mg, 0.93 mmol, 1 equiv.) and DMA (355 µL, 2.80 mmol, 3 equiv.) were dissolved in dry THF (1.86 mL) and then was added dropwise to a stirred solution of PhAcCl in dry THF (0.65 mL) at 0 °C.Then, the reaction mixture was allowed to reach RT and was stirred for 4 h. Thereafter, the crude mixture was put in aq. 5% HCl and the formed precipitate was filtered and subsequently washed with deionized water and a mixture of Et_2O-heptane (50 : 50, v/v) to give after drying under vacuum the bis-phenylacetamide **SD341** as a brown solid (260 mg, yield 62%). ^{1}H NMR (300 MHz, DMSO-d_6): 10.20 (s, 1H, N*H*), 10.15 (s, 1H, N*H*), 7.34-7.19 (m, 16H), 6.62 (dd, *J* 2.0 and 1.0, 1H), 3.60 (s, 2H), 3.58 (s, 2H), 2.10 (s, 1H) ppm; HPLC (system A): t_R = 5.3 min, purity = 97% (at 260 nm); LRMS (ESI+): *m/z* 451.3 (100) $[M + H]^+$, calcd for $C_{29}H_{27}N_2O_3^+$ 451.5.

6-Iminoxanthene-3-amine[27] *(SD338 / 339, N° CAS : 53164-45-7)*

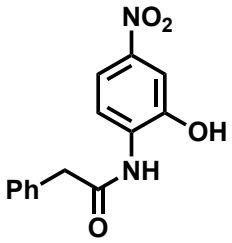


Bis-aniline **SD336** (200 mg, 0.93 mmol, 1 equiv.) was dissolved in 1,4-dioxane (2.5 mL) (or aq. 5% HCl) and DDQ was added. The resulting reaction mixture was stirred at RT for 3 h. Thereafter, the crude mixture was put in aq. 1.0 M NaOH and extracted with EtOAc. The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated under vacuum. The resulting residue was purified by semi-preparative RP-HPLC (system G, t_R = 18.7-23.1 min). The product-containing fractions were lyophilized to give the TFA salt of pyronin **SD338** / **339** as a dark purple amorphous powder (7 mg, yield 3%). ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.78 (s, 1H), 8.15 (s, 4H, N*H*), 7.84 (d, *J* 10.0, 2H), 7.00 (dd, *J* 2.0 and 9.0, 2H), 6.79 (d, *J* 2.0, 2H) ppm; HPLC (system A): t_R = 2,8 min, purity = 96% (at 260 nm); LRMS (ESI+): *m/z* 211.2 (100) $[M + H]^+$, calcd for $C_{13}H_{11}N_2O^+$ 211.2.

Highlighted Compounds of chapter 3

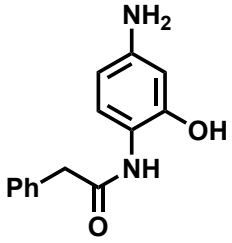
N-(2-Hydroxy-4-nitrophenyl)benzeneacetamide[28] *(SD325, N° CAS : 219664-51-4)*

[26] Adapted from : K. Arakawa, M. Inamasu, M. Matsumoto, K. Okumura, K. Yasuda, H. Akatsuka, S. Kawanami, A. Watanabe, K. Homma and Y. Saiga, *Chem. Pharm. Bull.*, **1997**, (45), 1984-1993.
[27] Unwanted but predictable reaction from: a) M. S. Mane, R. S. Balaskar, S. N. Gavade, P. N. Pabrekar, M. S. Shingare and D. V. Mane, *Chin. Chem. Lett.*, **2011**, (22), 1039-1042 ; b) B. Lal, R. M. Gidwani, J. Reden and N. J. De Souza, *Tetrahedron Lett.*, **1984**, (25), 2901-2904.
[28] M. I. Youshko, T. A. Shamolina, D. F. Guranda, A. V. Synev and V. K. Svedas, *Biochemistry (Moscow)*, **1998**, (63), 1104-1109.



2-Amino-5-nitrophenol (1.20 g, 7.78 mmol, 1 equiv.**)** and DMA (1.38 mL, 10.9 mmol, 1.4 equiv.) were dissolved in dry THF (3 mL) and then added dropwise to a solution of PhAc (1.03 mL, 7.78 mmol, 1 equiv.) in dry THF (15 mL) at 0 °C. Then, the mixture was allowed to reach RT and was stirred for 1 h. Thereafter, the crude mixture was diluted in aq. 5% HCl and the formed precipitate was filtered and subsequently washed with deionized water, Et_2O and heptane. The recovered solid was dried at 80 °C to give the phenylacetamide **SD325** as a yellowish solid (1.93 g, 91%). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 11.05 (s, 1H, N*H*), 9.63 (s, 1H, O*H*), 8.29 (d, *J* 9.0, 1H), 7.70 (d + s, *J* 10.8, 2H), 7.34-7.25 (m, 5H), 6.89 (d, *J* 8.1, 1H), 3.85 (s, 2H) ppm.

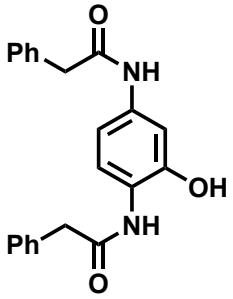
N-(4-Amino-2-hydroxyphenyl)benzeneacetamide[16,29] *(SD329, N° $_{CAS}$: 865837-39-4)*



Nitro-aryl **SD325 (**1,8 g, 6.6 mmol, 1 equiv.**)** and $SnCl_2 . H_2O$ (6.0 g, 26.4 mmol, 4 equiv.**)** were dissolved in EtOAc (45 mL) and the resulting reaction mixture was stirred at reflux (85 °C) for 5 h. A further amount of $SnCl_2 . H_2O$ (1.2 g, 0.8 equiv.) was added and the mixture was refluxed overnight. Again, a further amount of $SnCl_2 . H_2O$ (6.0 g, 4 equiv.) was added and refluxed further 2 h. The crude mixture was cooled and deionized water was added. The pH was adjusted to 8 with aq. 10% NaOH (Sn salts underwent slow hydrolysis and the pH variation needs to be monitored). Then, this aq. mixture was extracted with EtOAc. The organic layer was washed with deionized water, brine, dried over anhydrous Na_2SO_4, filtered and concentrated under vacuum. The resulting residue was dissolved in hot $CHCl_3$ for 1 h and filtered hot. Filtrate was concentrated under vacuum and the resulting solid was washed with $CHCl_3$-Et_2O to obtain a pale grey solid (75.9 mg). The filtrate was concentrated and resuspended/sonicated in $CHCl_3$-Et_2O and then filtrate to obtain a second batch as a purple solid (840 mg). The two batches (915 mg, yield 57%) were pure enough to be used in the next step. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 9.30 (s, 1H), 9.24 (s,1H), 7.35-7.22 (m, 5H), 7.12 (d, *J* 8.5, 1H), 6.10 (d, *J* 2.0, 1H), 5.99 (dd, *J* 2.0 and 8.5, 1H), 4.86 (bs, 2H, N*H*), 3.64 (s, 2H) ppm; HPLC (system A): t_R = 2.4 min, purity > 94% (at 260 nm); LRMS (ESI+): *m/z* 243.1 (100) $[M + H]^+$, calcd for $C_{14}H_{15}N_2O_2^+$ 243.3; LRMS (ESI-): *m/z* 240.8 (100) $[M - H]^-$, calcd for $C_{14}H_{13}N_2O_2^-$ 241.3.

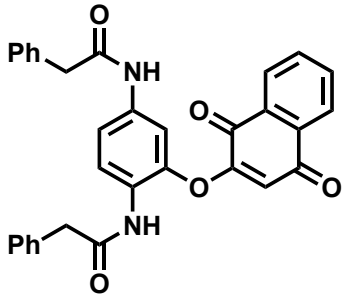
[29] The reduction by cat. hydrogenation (with Pd / C) didn't work.

Bis-phenylacetamide derivative *(SD331)*



Aniline **SD329 (**200 mg, 0.83 mmol, 1 equiv.**)** and DMA (156 µL, 1.24 mmol, 1.5 equiv.) were dissolved in dry THF (0.3 mL) and the solution was added dropwise to a solution of PhAcCl (110 µL, 0.83 mmol, 1 equiv.) in dry THF (2 mL) at 0 °C. Then, the mixture was allowed to reach RT and was stirred for 1 h. Thereafter, the crude mixture was diluted in aq. 5% HCl and the formed precipitate was filtered and subsequently washed with deionized water, Et_2O and heptane. The light violet solid obtained (190 mg, yield 64%) is pure enough to be used in the next step. For analytical purpose, a part of the solid was washed with EtOAc and MeOH and a light pink / white solid was finally obtained. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 10.04 (s, 1H, N*H*), 9.87 (s, 1H, O*H*), 9.28 (s, 1H, N*H*), 7.63 (d, *J* 8.7, 1H), 7.33-6.23 (m, 11H), 6.89 (d, *J* 8.1, 1H), 3.71 (s, 2H), 3.60 (s, 2H) ppm; HPLC (system A): t_R = 4.4 min, purity = 100% (at 260 nm); LRMS (ESI+): *m/z* 361.2 (100) $[M + H]^+$, calcd for $C_{22}H_{21}N_2O_3^+$ 361.4; LRMS (ESI-): *m/z* 358.9 (100) $[M - H]^-$, calcd for $C_{22}H_{19}N_2O_3^-$ 359.41.

Mixed bis-aryl ether[30] *(SD335)*

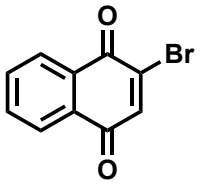


Phenol **SD331** (60 mg, 0.17 mmol, 1 equiv.) and anhydrous K_2CO_3 (25 mg, 0.17 mmol, 1.05 equiv.) were dissolved in MeOH (1 mL)**.** After 1 h, **SD306** (*vide infra*, 40 mg, 0.17 mmol, 1 equiv.) was added and the mixture was stirred at RT for 3 h. Thereafter, the crude mixture was diluted in EtOAc and washed with aq. 5% $NaHCO_3$. The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated under vacuum. The resulting residue was purified by chromatography on a silica gel column (eluent: pentane-EtOAc, a step gradient from 3 : 7 to 0 : 10, v/v) to give the desired product but not completey pure (31 mg, yield 40%). A further purification by semi-preparative RP-HPLC (system H, t_R = 12.6-13.5 min) was performed. The product-containing fractions were lyophilized to give the PGA probe **SD335** as light

[30] The synthetic method based on the use of methanolic potassium hydroxyde (see: A. Grzelakowska, J. Kolinska, M. Zaklos-Szyda, R. Michalski and J. Sokolowska, *Color. Technol.*, **2017**, (133), 145-157) was found only after this experiment was over. If another reaction of this type is launched, this method must be considered.

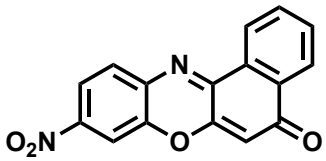
brown solid (0.8 mg, yield 1%). R_f (Heptane-EtOAc, 1 : 2, v/v) = 0.62; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 10.31(s, 1H, N*H*), 10.02 (s, 1H, N*H*), 7.98-6.75 (m[31], 18H), 5.29 (s,1H), 4.56 (s, 1H), 3.67 (s, 2H) ppm; the amount was too low to perform IR analysis. HPLC (system A): t_R = 5.3 min, purity = 97% (at 260 nm); LRMS (ESI+): *m/z* 517.0 (100) $[M + H]^+$, calcd for $C_{32}H_{25}N_2O_5$ 517.5 and *m/z* 558.0 (80) $[M + H + CH_3CN]^+$, calcd for $C_{34}H_{28}N_3O_5^+$ 558.5; LRMS (ESI-): *m/z* 514.9 (100) $[M - H]^-$, calcd for $C_{32}H_{23}N_2O_5^-$ 515.5 and *m/z* 560.6 (30) $[M - H + FA]^-$, calcd for $C_{33}H_{25}N_2O_7^-$ 561.5.

2-Bromo-1,4-naphthoquinone[32] *(SD306, N° CAS : 2065-37-4, commercial chemical cmpd)*



N-Bromosuccinimide (3.70 g, 20.8 mmol, 2 equiv.) was dissolved in glacial AcOH (100 mL) and deionized water (200 mL) and heated at 65 °C. A solution of 1-naphthol (1.50 g, 10.4 mmol, 1 equiv.) in glacial AcOH (100 mL) was added over 1 h and the reaction mixture was stirred for further 30 min. Thereafter, the crude mixture was diluted in deionized water and extracted with $CHCl_3$. The organic layer was washed with aq. 5% $NaHCO_3$ (CO_2 release), brine, dried over anhydrous $MgSO_4$, filtered and concentrated under vacuum. The resulting residue was recrystallized in absolute EtOH. The precipitate was filtered to recover a first fraction (brown solid, 537 mg). The filtrate was concentrated and purified by chromatography on a silica gel column (eluent: heptane-EtOAc, a step gradient from 10 : 0 to 9 : 1, v/v). A further trituration of solid in absolute EtOH afforded a second fraction as a yellow / brown solid (231 mg) (768 mg, combined yield 31%). Experimental spectra (NMR, MS, ...) similar to those previously reported in the literature[32].

9-Nitro-5*H*-benzo[*a*]phenoxazin-5-one[33] *(SD309, N° CAS : 75197-93-2)*



2-Amino-5-nitrophenol (65 mg, 0.42 mmol, 1 equiv.) and anhydrous K_2CO_3 (62 mg, 0.44 mmol, 1.05 equiv.) were dissolved in dry DMF (10 mL) and the resulting mixture was stirred at RT for 1 h. Then, 2-bromo-1,4-naphthoquinone **SD306** (100 mg, 0.42

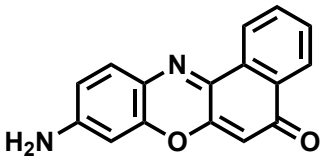
[31] The approximate integration value of each pic doesn't fit with the expected value which appears when the whole aromatic range is fully integrated. Another point of the spectra is one of the CH_2 goes out with two deshielding signals and the other one with one "normal" signal. This is supported by ^{1}H NMR ($CDCl_3$) spectrum of impure compound where the two CH_2 appears as two "normal" signal.

[32] a) T. V. Nguyen and N. De Kimpe, *Tetrahedron*, **2003**, (59), 5941-5946 ; b) M. A. Brimble, P. Bachu and J. Sperry, *Synthesis*, **2007**, 2887-2893 ; c) S. A. Chitre, G.-A. M. Lobo, S. M. Rathod, R. B. Smith, R. Leslie, C. Livingstone and J. Davis, *J. Chromatogr. B* **2008**, (864), 173-177 ; d) P. Bachu, J. Sperry and M. A. Brimble, *Tetrahedron*, **2008**, (64), 4827-4834 ; e) V. S. Khodade, A. T. Dharmaraja and H. Chakrapani, *Bioorg. Med. Chem. Lett.*, **2012**, (22), 3766-3769.

[33] a) N. L. Agarwal and W. Schaefer, *J. Org. Chem.*, **1980**, (45), 5144-5149 ; b) J. Nakanishi, T. Nakajima, M. Sato, T. Ozawa, K. Tohda and Y. Umezawa, *Anal. Chem.*, **2001**, (73), 2920-2928.

mmol, 1 equiv.) was added and the resulting mixture was stirred for 2 h[34]. Thereafter, the crude mixture was diluted in aq. 1.0 M HCl / ice. The formed precipitate was filtered to recover 9-nitro-5*H*-benzo[*a*]phenoxazin-5-one as **a** brown solid (36 mg, yield 30%). Experimental spectrum (NMR) similar to those previously reported in the literature[33].

9-Amino-5*H*-benzo[*a*]phenoxazin-5-one[33b] *(SD340, N° $_{CAS}$: 13456-56-9)*



Nitro-aryl derivative **SD309** (36 mg, 0.116 mmol, 1 equiv.) was dissolved in MeOH (1 mL) under Ar. Atmosphere. Then, Pd / C 5% (12 mg, 0.05 equiv.) was added and the mixture was put under H_2 atmosphere for 2 h 30. The reaction mixture was dissolved in pyridine and filtered over a Dicalite® pad (to remove Pd / C). The filtrate was diluted with deionized water to initiate precipitation. The precipitate was filtered and retaken in a mixture of CH_3CN, aq. (0.1%) TFA (5%) and DMSO (5%) and then was centrifuged. After decantation, the solid residue was suspended in deiinized water and then centrifuged again before freeze-drying. The desired fluorophore (TFA salt) was obtained as a black solid (20 mg, yield 46%). HPLC (system A): t_R = 6.2 min, purity = 96% (at 260 nm). Experimental spectra (NMR, MS, ...) similar to those previously reported in the literature[33b].

[34] HPLC-MS analyses clearly showed that the progress of the reaction was quitely the same after 10 min and 2 h.

SI.2. Published experimental data

SI.2.1. Experimental section and "Supporting Information" file of Article 1: Dual enzyme-responsive "turn-on" fluorescence sensing systems based on in situ formation of 7-hydroxy-2-iminocoumarin scaffolds

Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

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

Sylvain Debieu,[a] and Anthony Romieu[*a,b]

[a] *Institut de Chimie Moléculaire de l'Université de Bourgogne, UMR 6302, CNRS, Univ. Bourgogne Franche-Comté, 9 Avenue Alain Savary, 21078 Dijon, France*
Fax: +33 (0)3 80 39 61 17
Tel: +33 (0)3 80 39 36 24
E-mail: anthony.romieu@u-bourgogne.fr
Lab homepage: http://www.icmub.fr

[b] *Institut Universitaire de France, 103 Boulevard Saint-Michel, 75005 Paris, France*

Supporting Information

Table of contents

Abbreviations

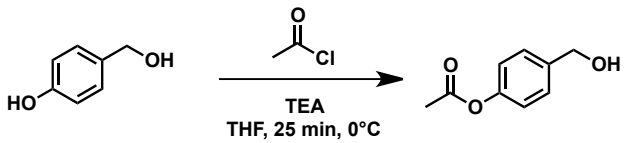
The following abbreviations are used throughout the text of the ESI file: Ar, argon; DCM, dichloromethane; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; equiv., equivalent(s); Et_2O, diethyl ether; EtOAc, ethyl acetate; EtOH, ethanol; ESI, electrospray ionisation; FA, formic acid; HPLC, high-pressure 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]

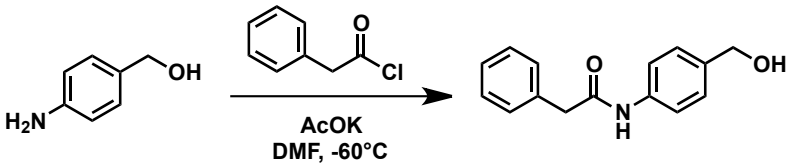


Under Ar atmosphere, 4-hydroxybenzyl alcohol (2 g, 16.1 mmol) was dissolved in dry THF (27 mL), cooled to 0 °C with an ice-water bath and TEA (2.23 mL, 16.1 mmol, 1 equiv.) was added. Then acetyl chloride (1.26 mL, 17.7 mmol, 1.1 equiv.) was added dropwise over a period of 25 min. The resulting reaction 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.

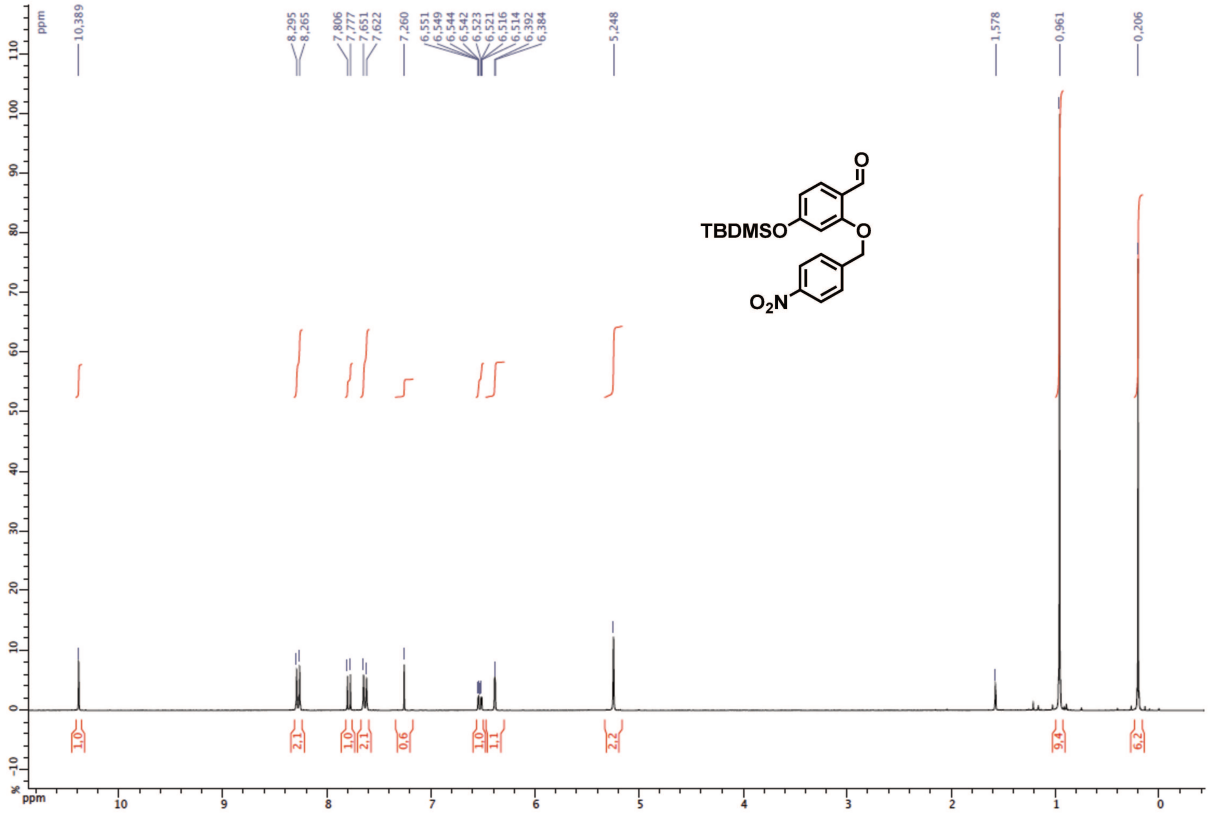
Sequential protocol (NTR then hydrolase):
39 nmol of PGA-NTR (23 µL of mixture A) (or PLE-NTR (20 µL of mixture B)) fluorogenic probe **9** (or **8**) was dissolved in PB (260 µL (or 428 µL)) containing 6 µL of mixture H (0.6 U) and 2 µL of mixture G and the resulting enzymatic reaction mixture was incubated 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.

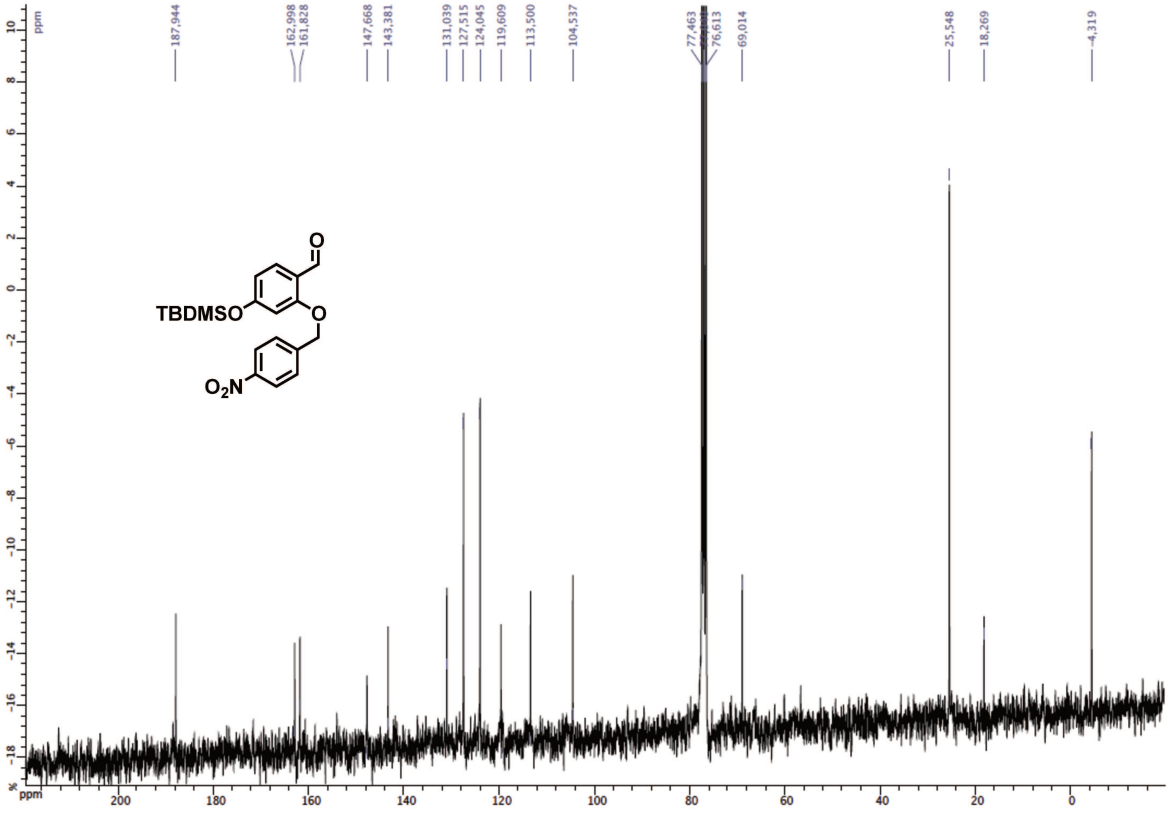
Samples treatment for HPLC-MS analysis:
Withdrawn sample (50 µL) was diluted with 50 µL of CH_3CN, then vortexed followed by centrifugation (9 000 rpm, 2 min) and finally, 75 µL of the supernatant was collected and diluted with 25 µL of aq. 0.1% FA. 10 µL was injected into the HPLC-MS apparatus (system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

Analytical data

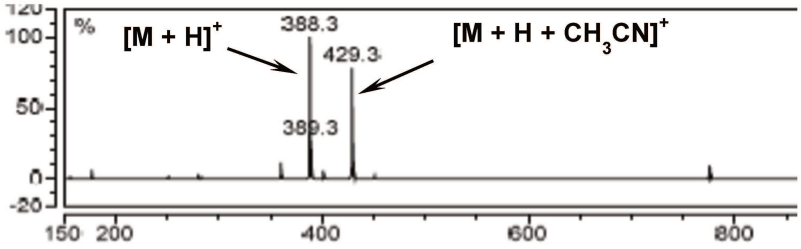
^{1}H NMR spectrum of compound 2 recorded in $CDCl_3$ at 300 MHz

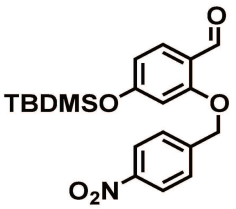


^{13}C NMR spectrum of compound 2 recorded in $CDCl_3$ at 75 MHz

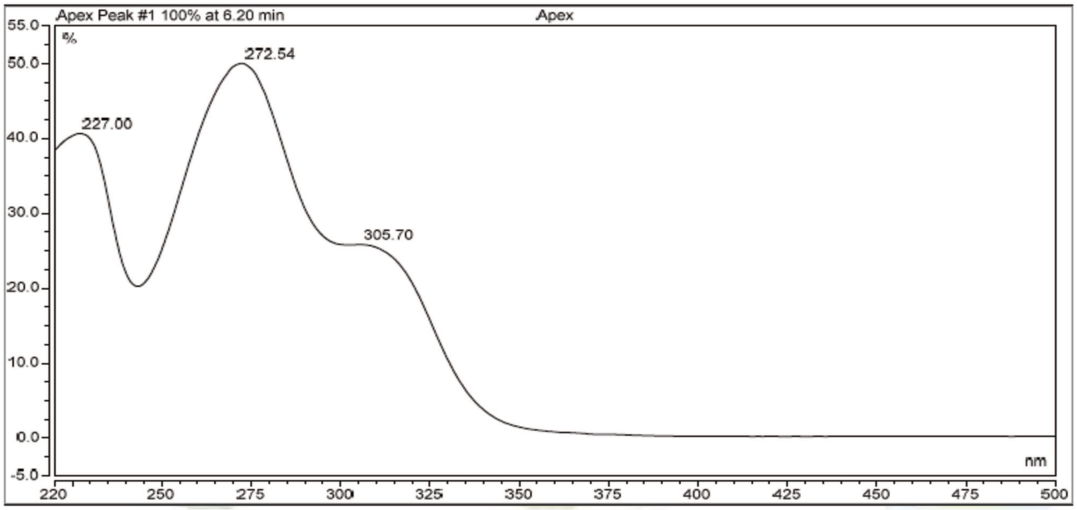


ESI+ mass spectrum (low resolution) and UV-vis spectrum of compound 2

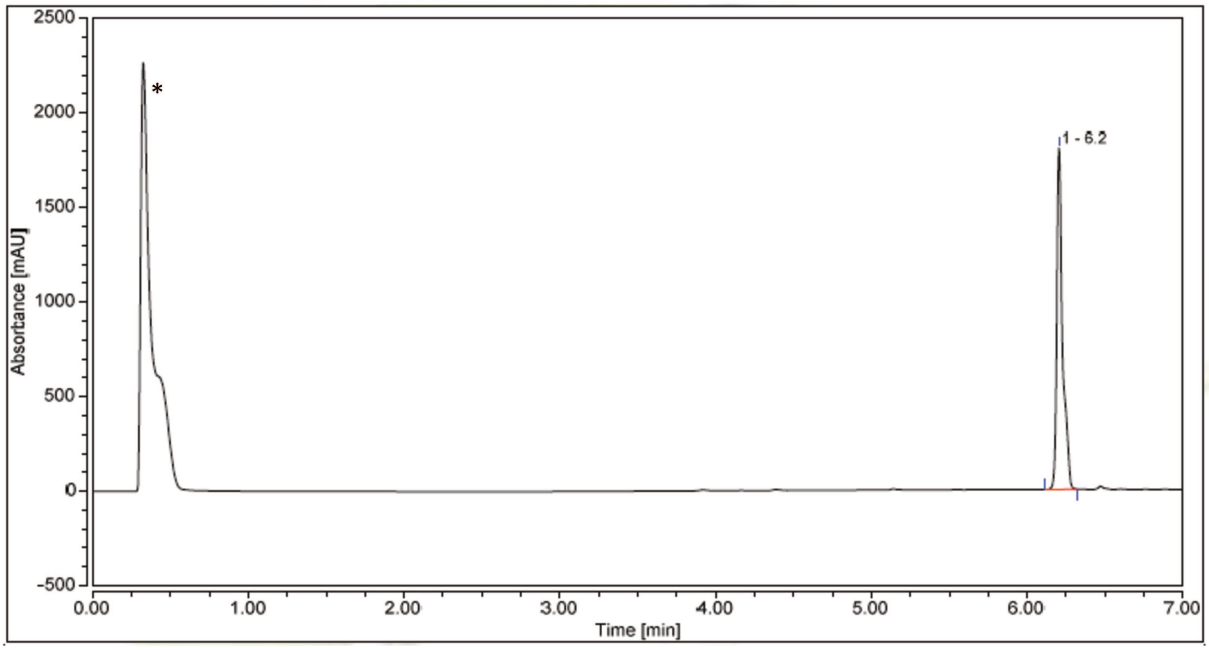




Chemical Formula: $C_{20}H_{25}NO_5Si$
Molecular Weight: 387,50

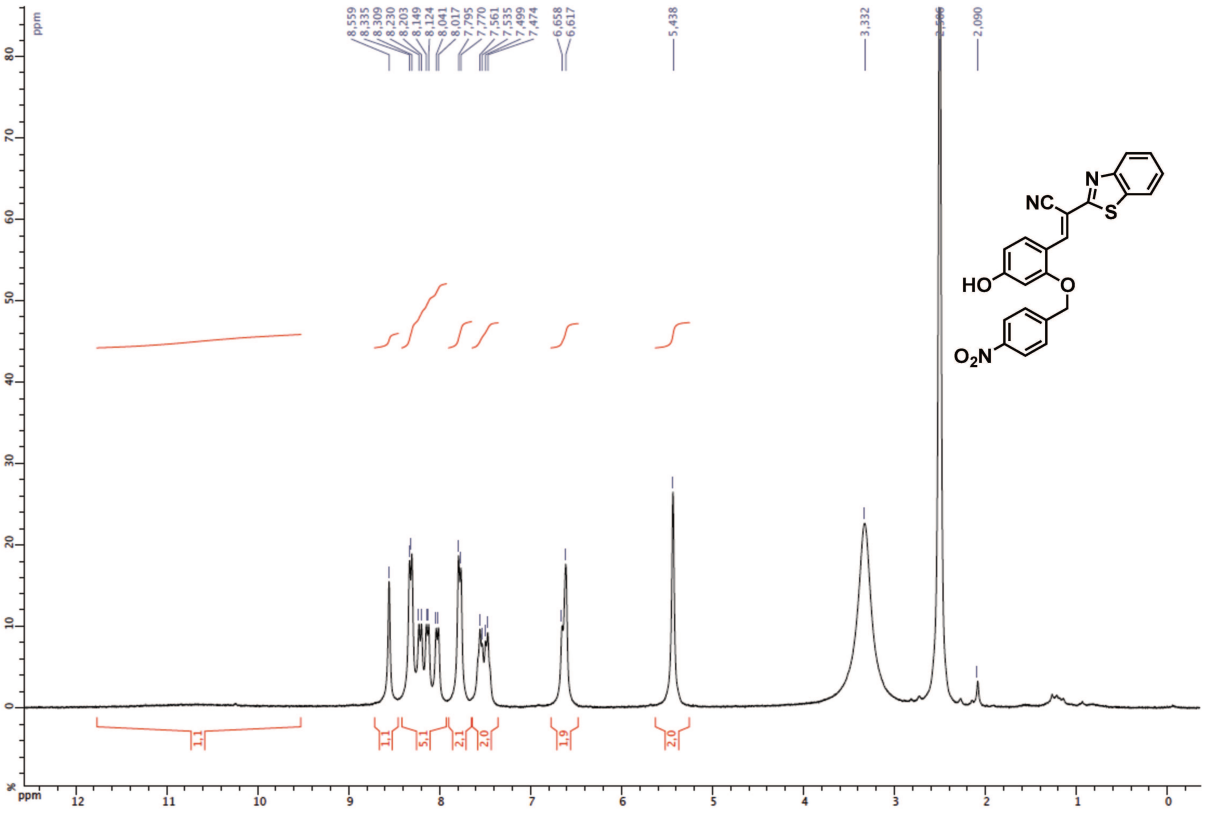


RP-HPLC elution profile (system A) of compound 2 at 260 nm

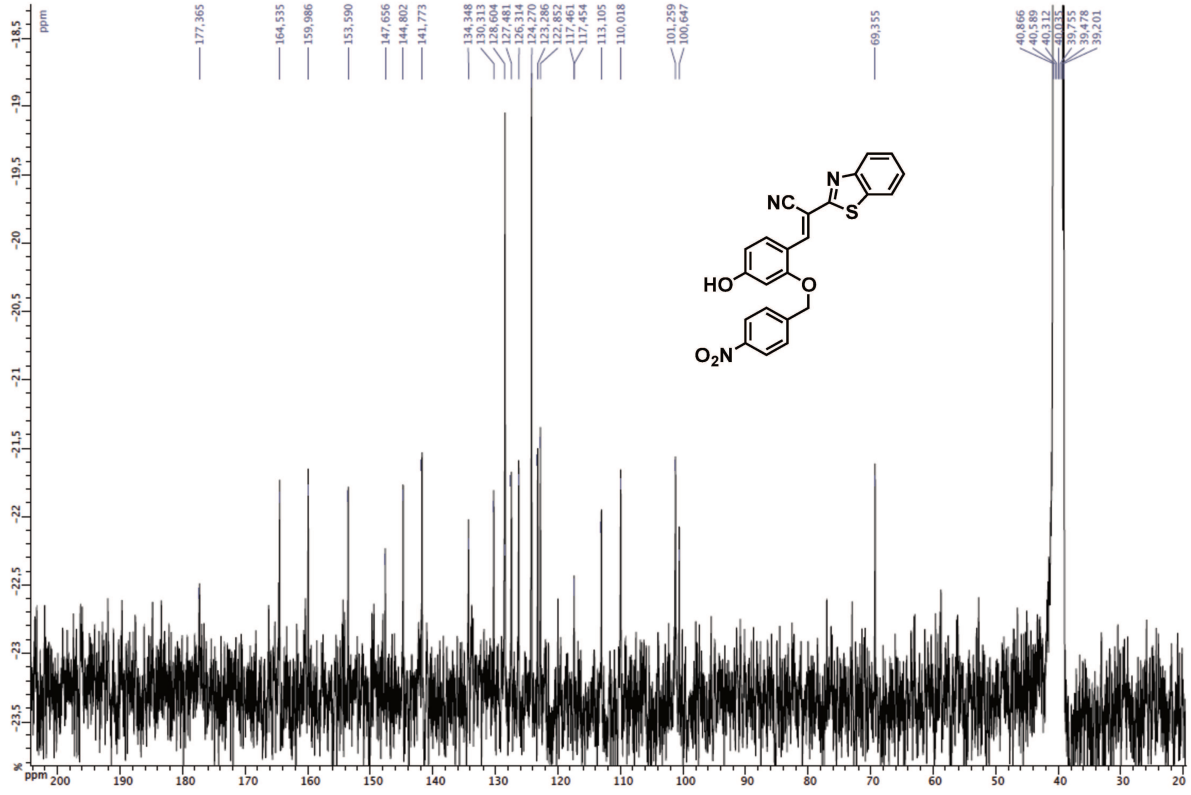


**peak assigned to acetone used as solvent for sample preparation*

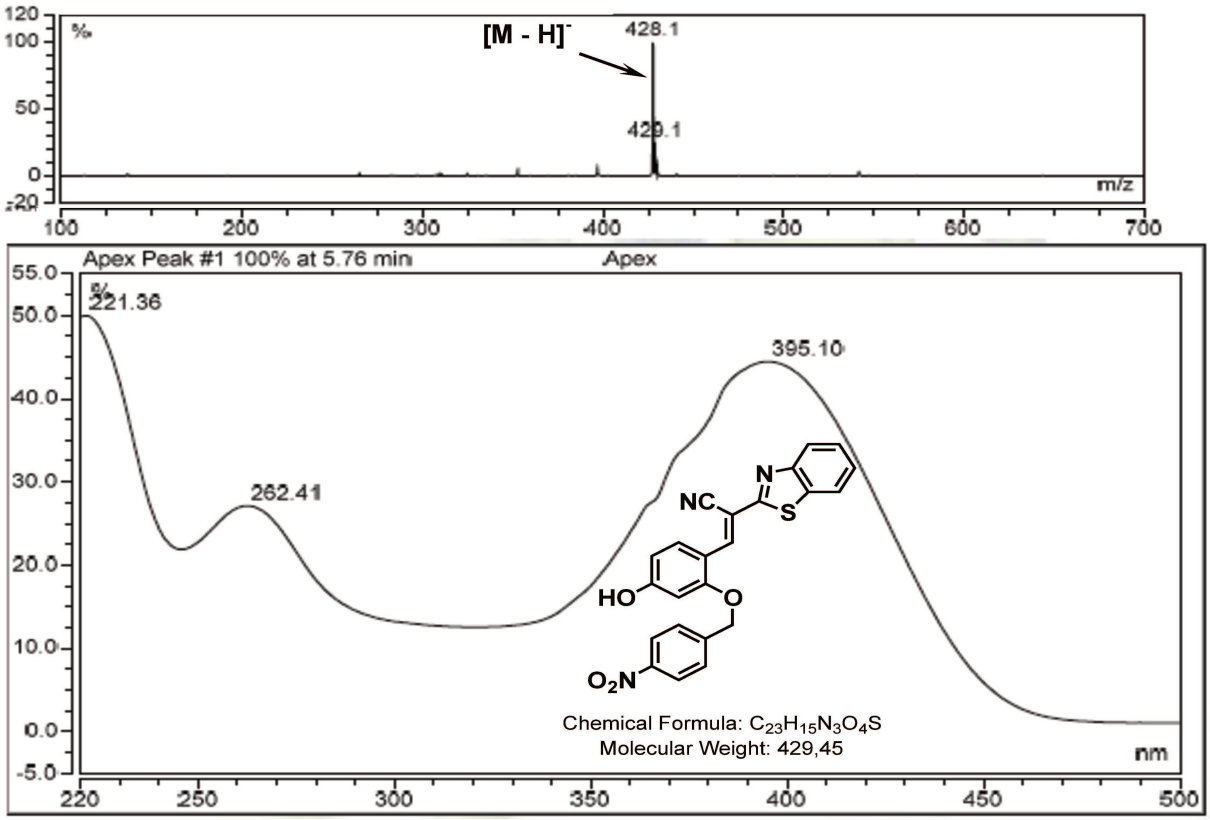
^{1}H NMR spectrum of compound 3 recorded in DMSO-d_6 at 300 MHz



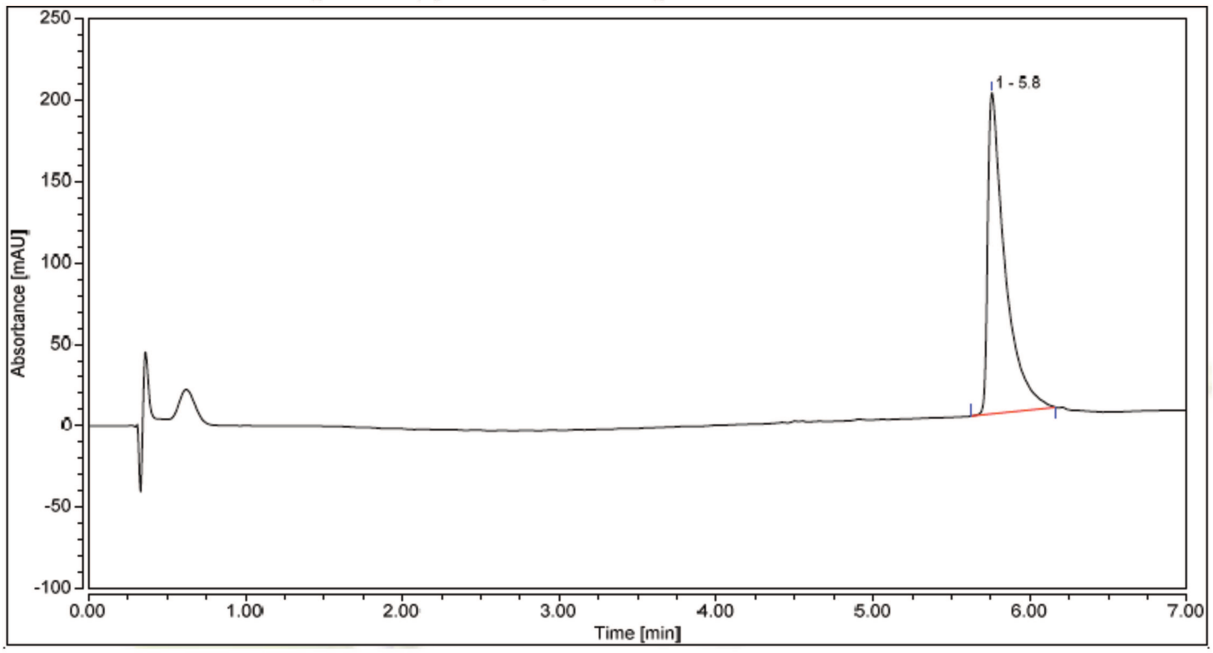
^{13}C NMR spectrum of compound 3 recorded in DMSO-d_6 at 75 MHz



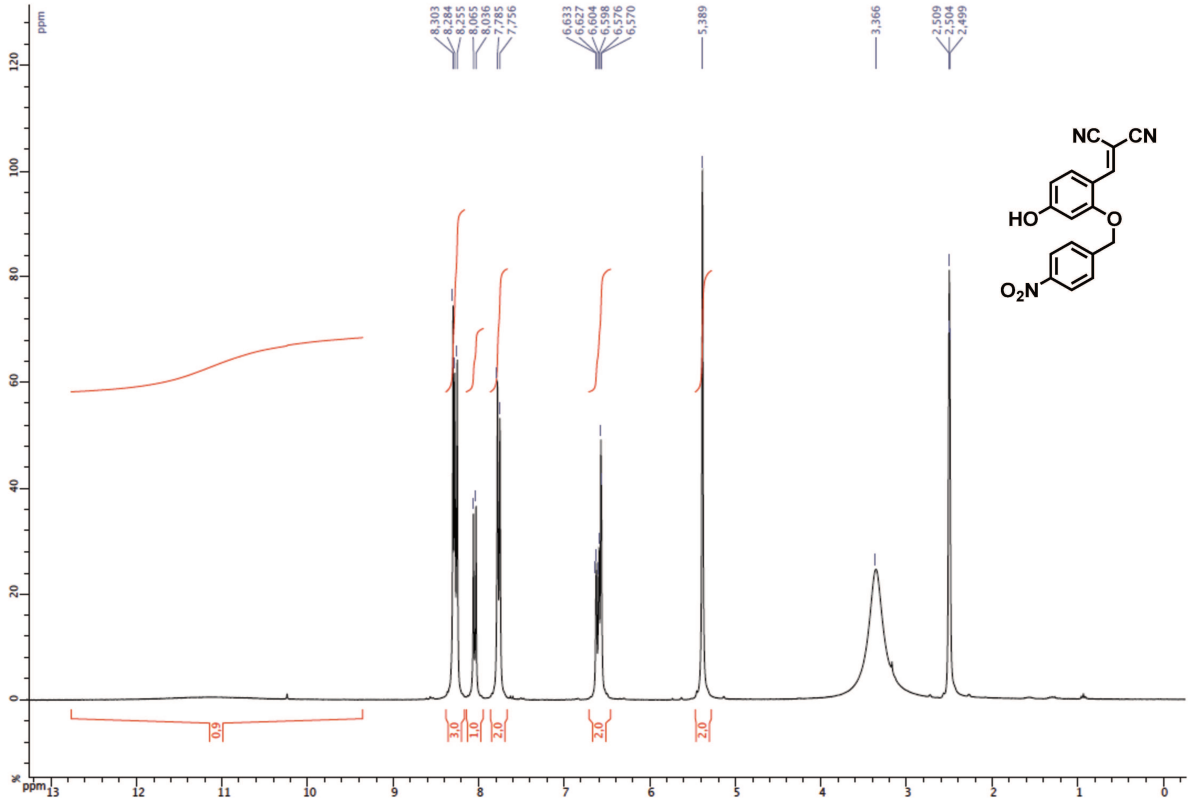
ESI- mass spectrum (low resolution) and UV-vis spectrum of compound 3



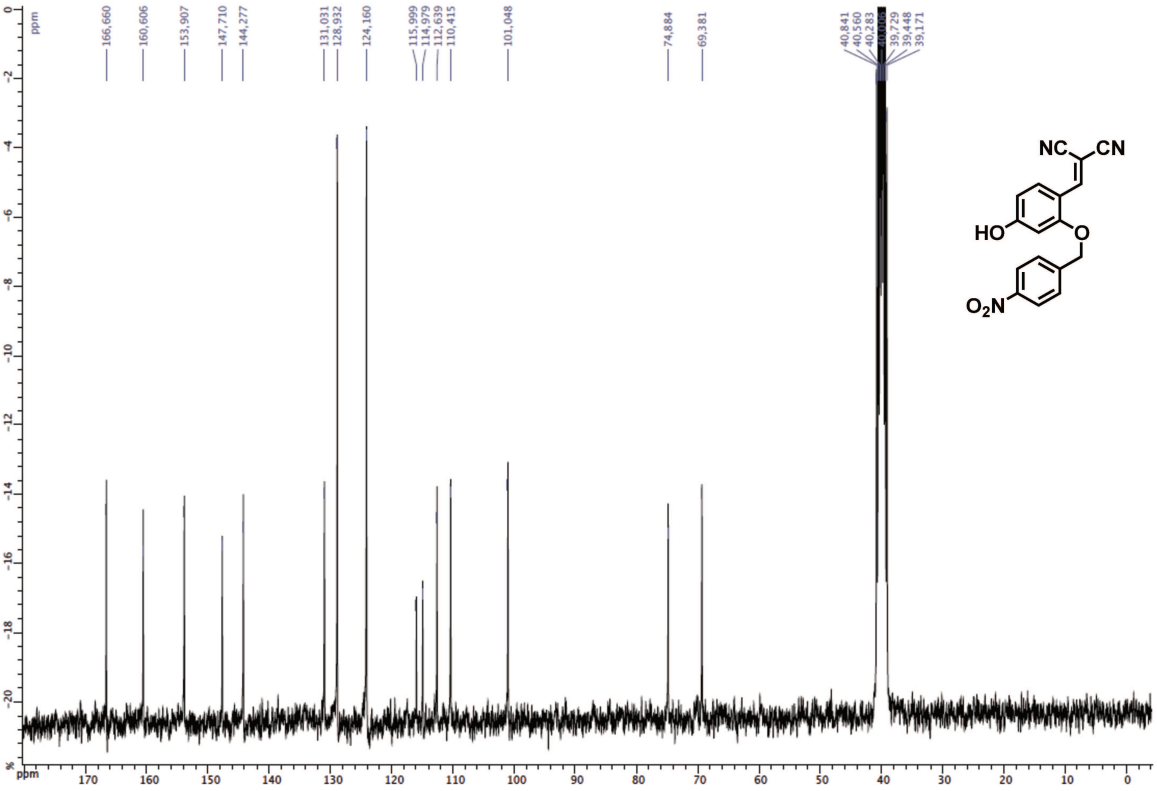
RP-HPLC elution profile (system B) of compound 3 at 260 nm



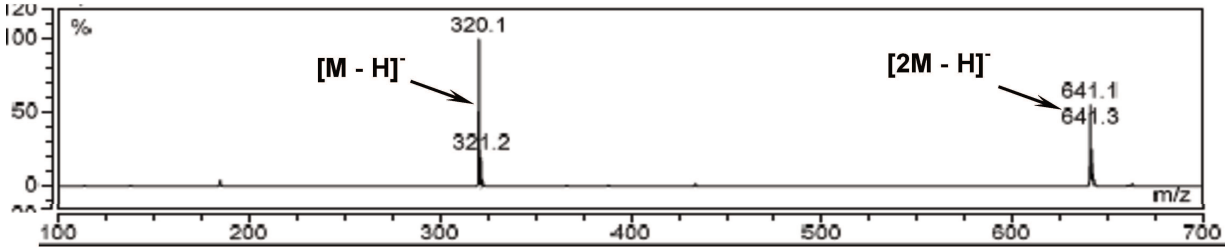
^{1}H NMR spectrum of compound 4 recorded in DMSO-d_6 at 300 MHz

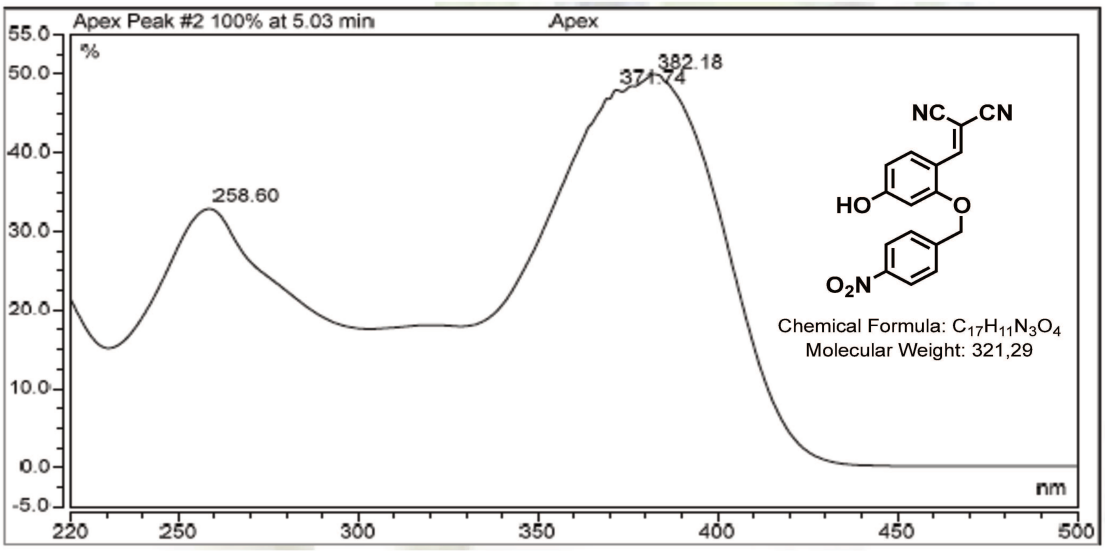


^{13}C NMR spectrum of compound 4 recorded in DMSO-d_6 at 75 MHz

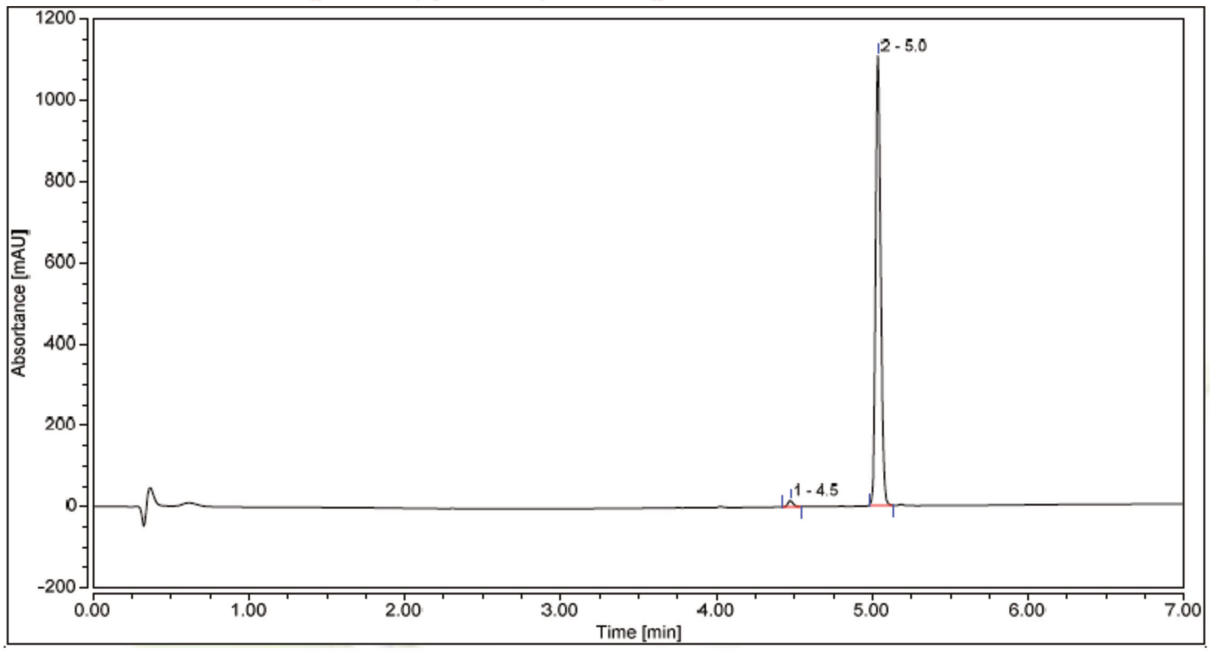


ESI- mass spectrum (low resolution) and UV-vis spectrum of compound 4

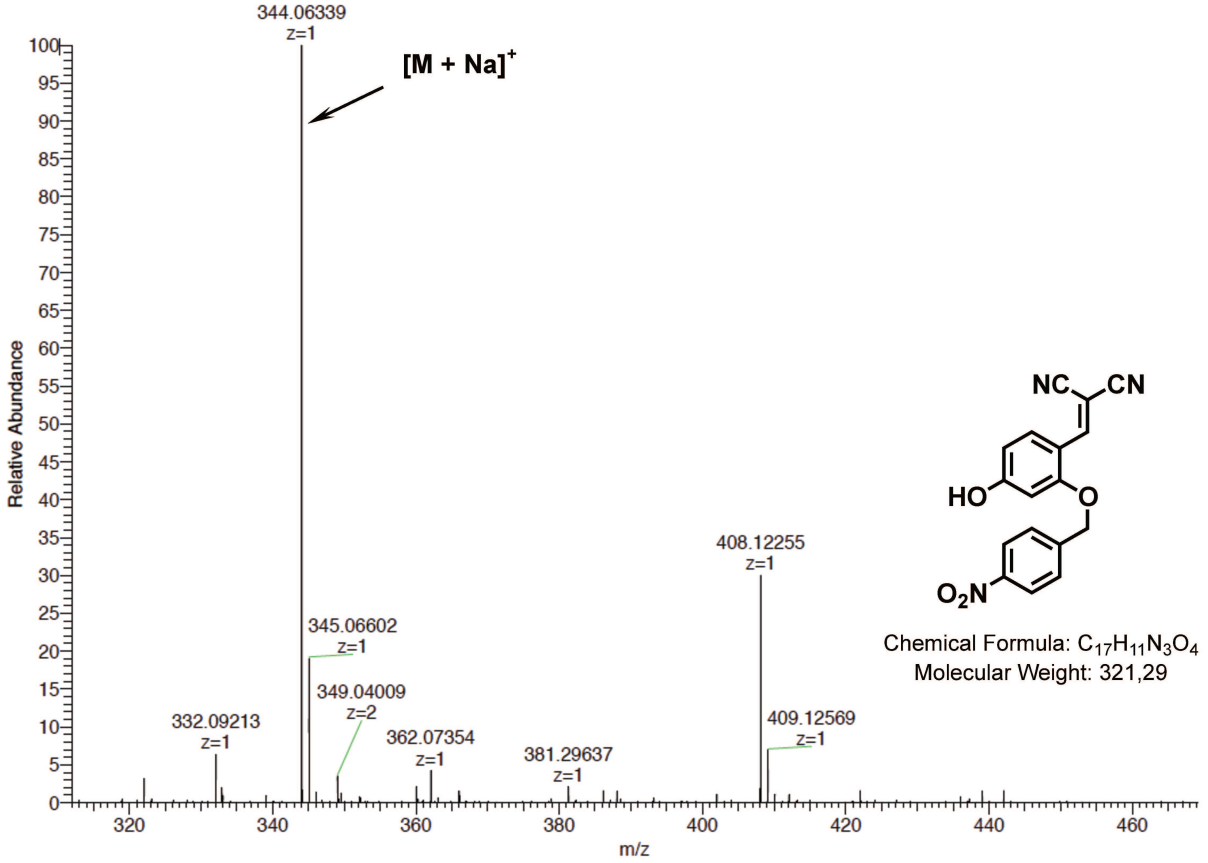


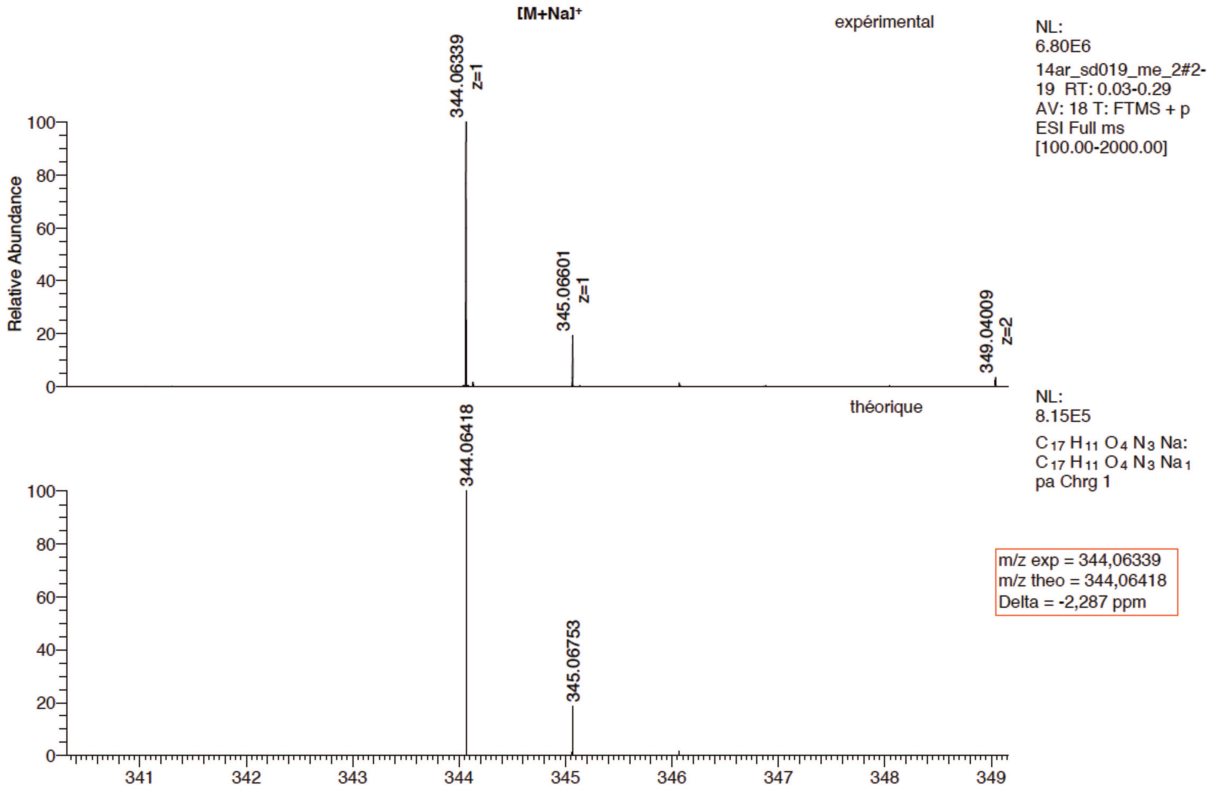


RP-HPLC elution profile (system B) of compound 4 at 260 nm

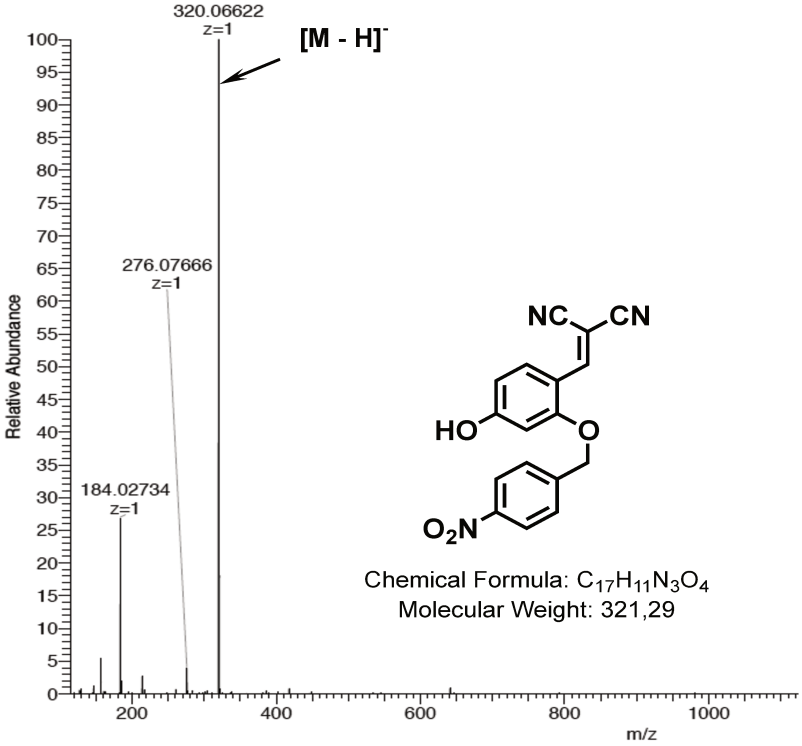


ESI+ mass spectrum (high resolution) of compound 4

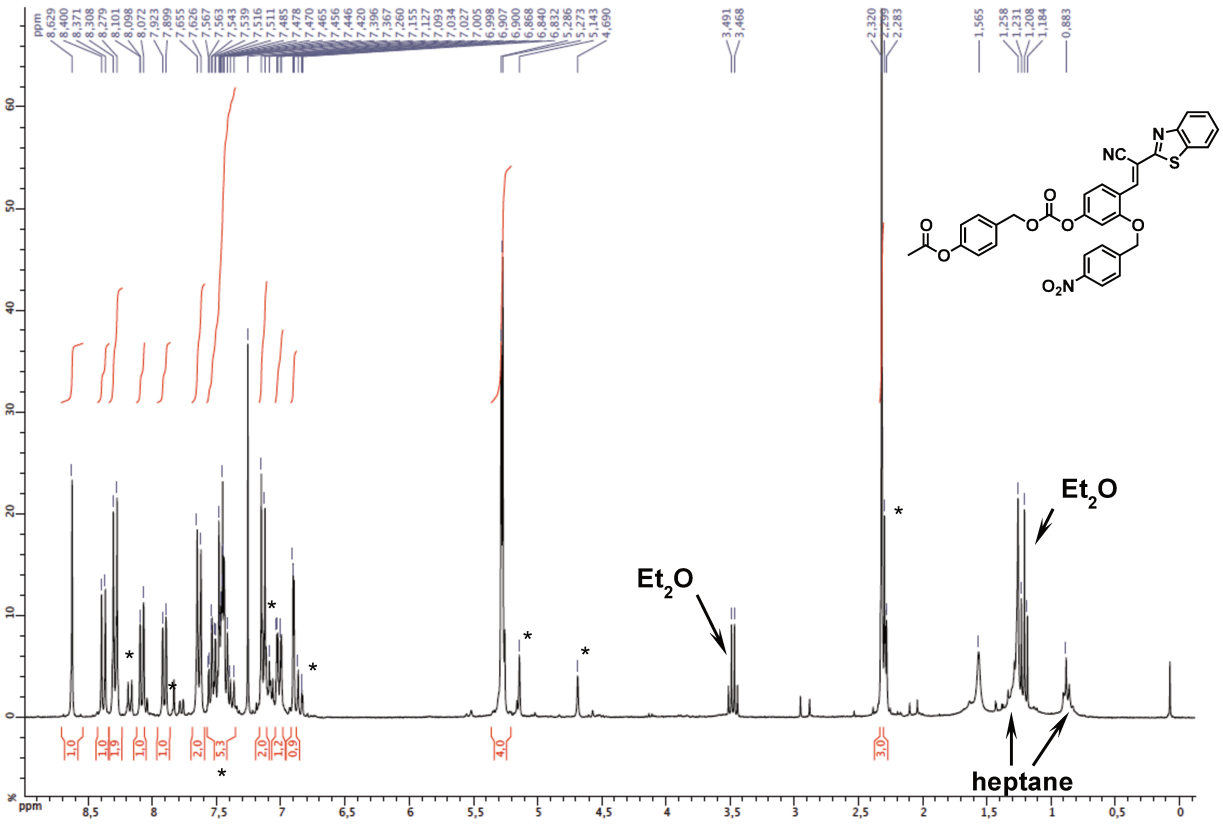




ESI- mass spectrum (high resolution) of compound 4

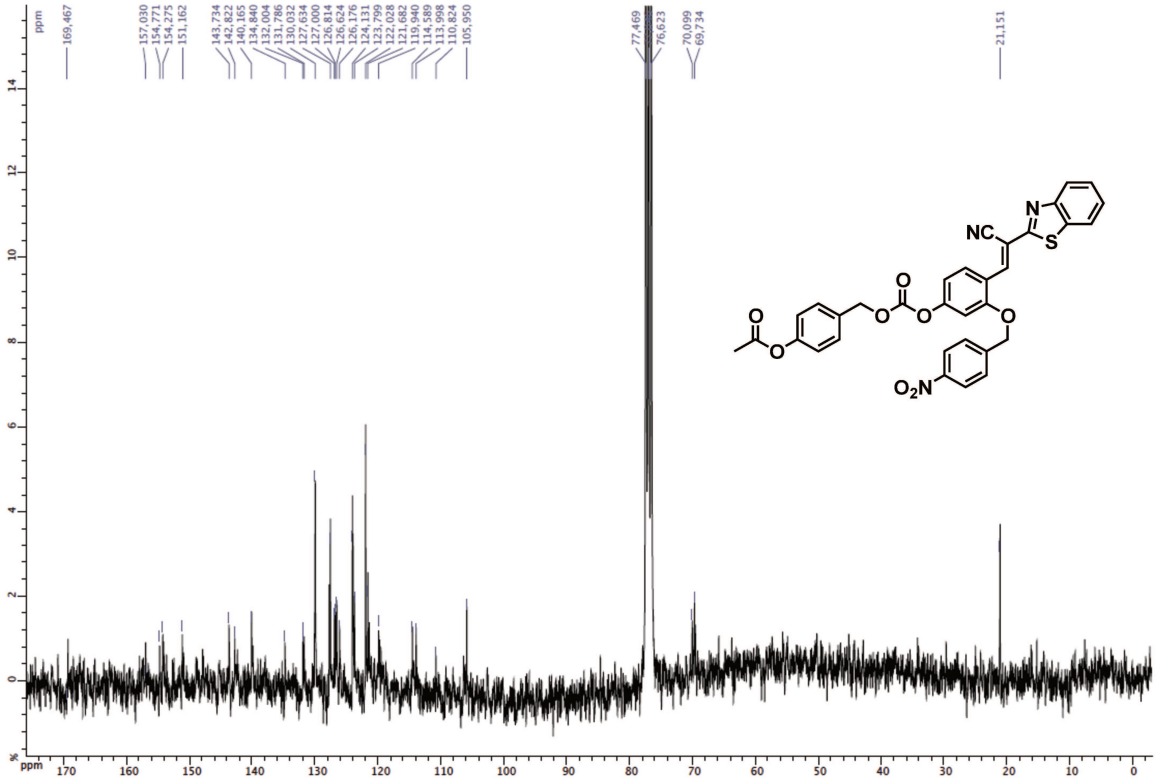


^{1}H NMR spectrum of compound 5 recorded in $CDCl_3$ at 300 MHz

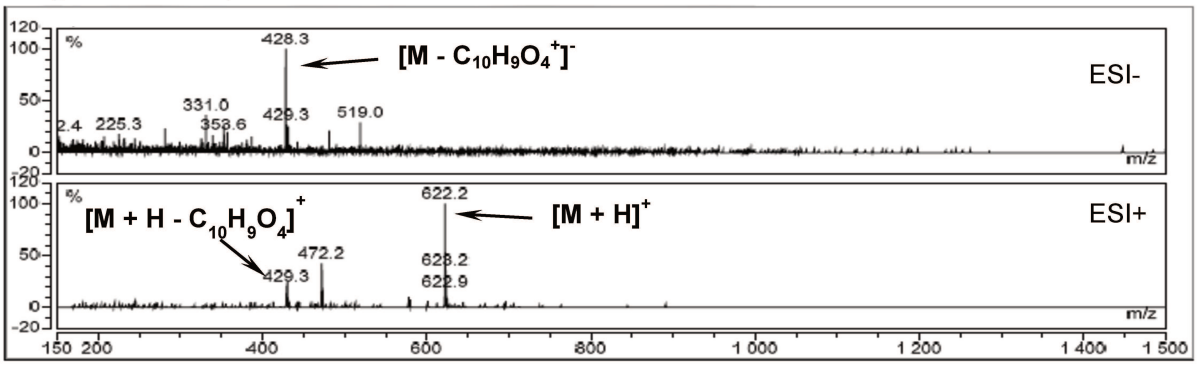


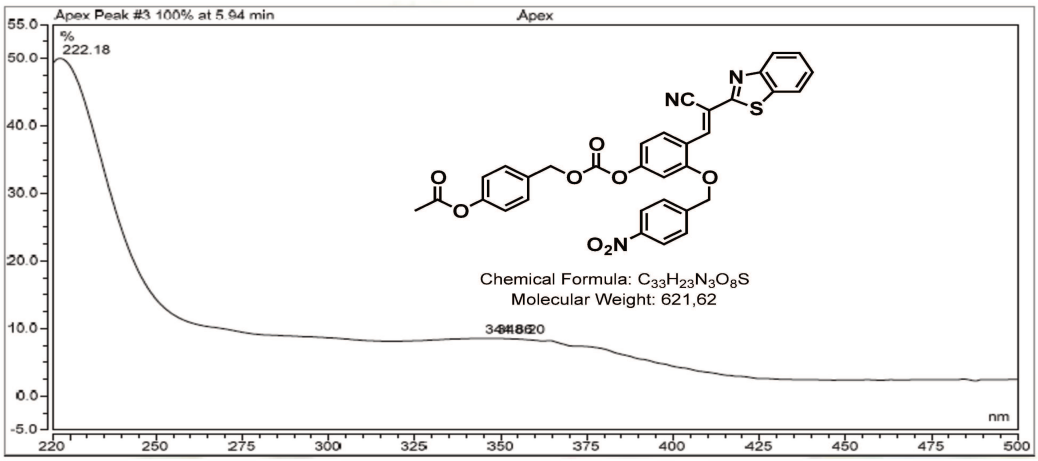
**peaks assigned to 2nd geometric isomer (15 : 85)*

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

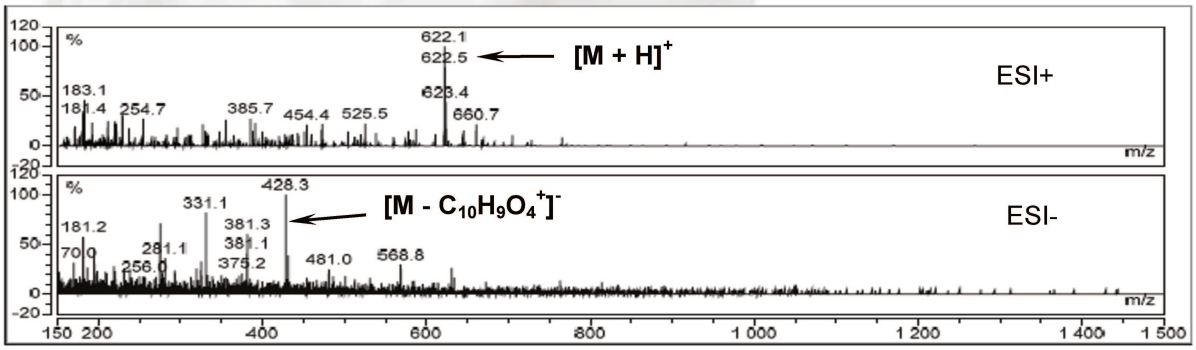


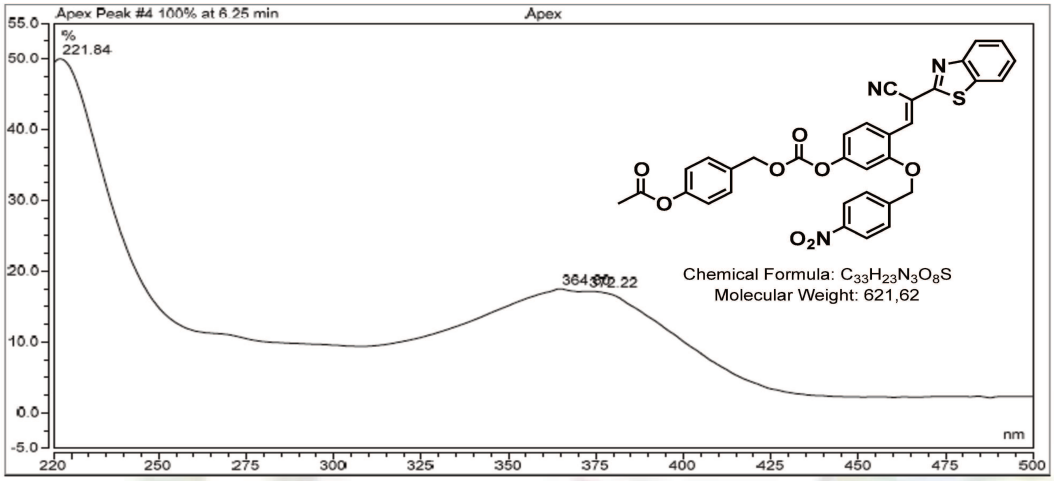
ESI-/ESI+ mass spectrum (low resolution) and UV-vis spectrum of compound 5 (more polar isomer)



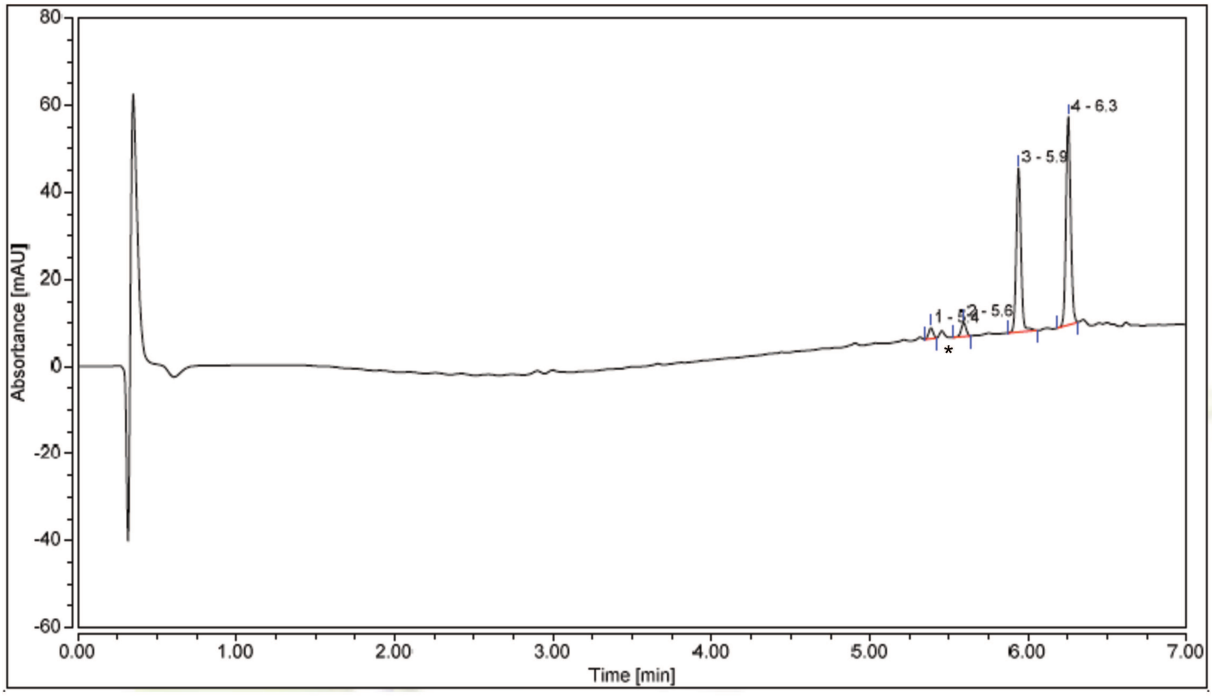


ESI-/ESI+ mass spectrum (low resolution) and UV-vis spectrum of compound 5 (less polar isomer)



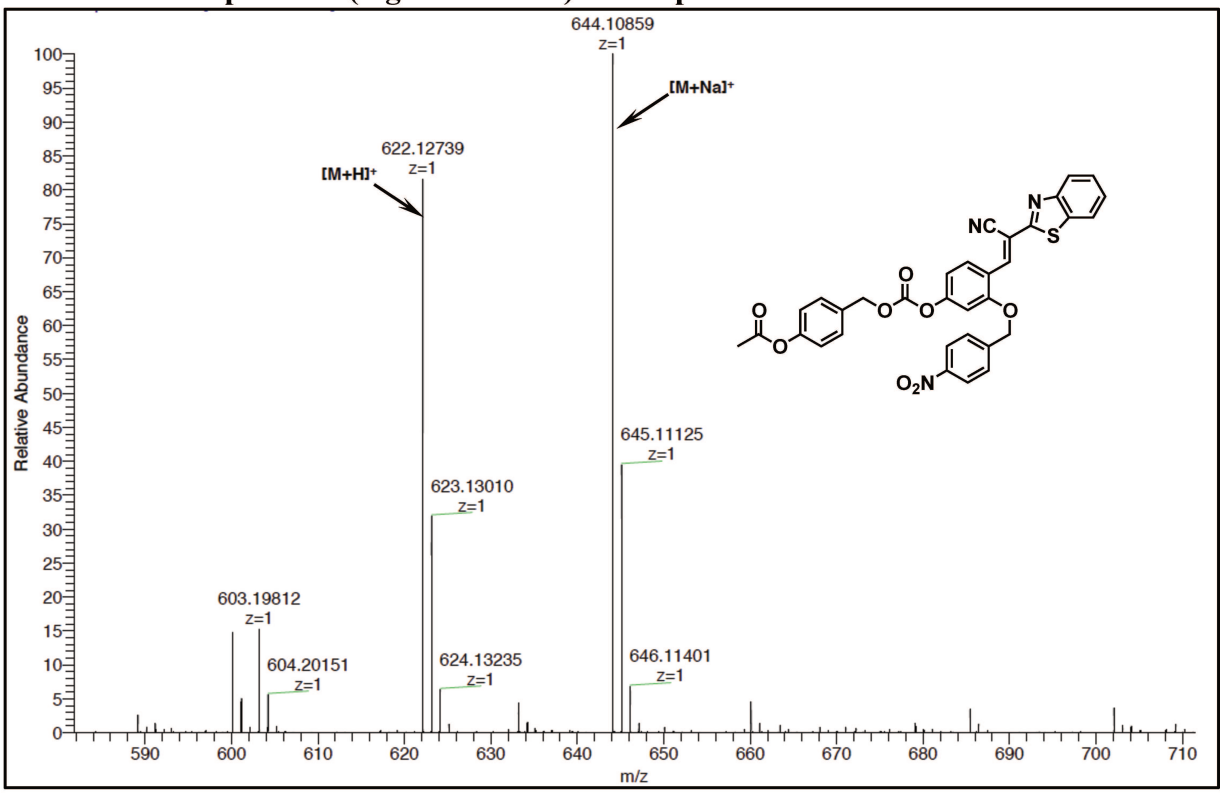


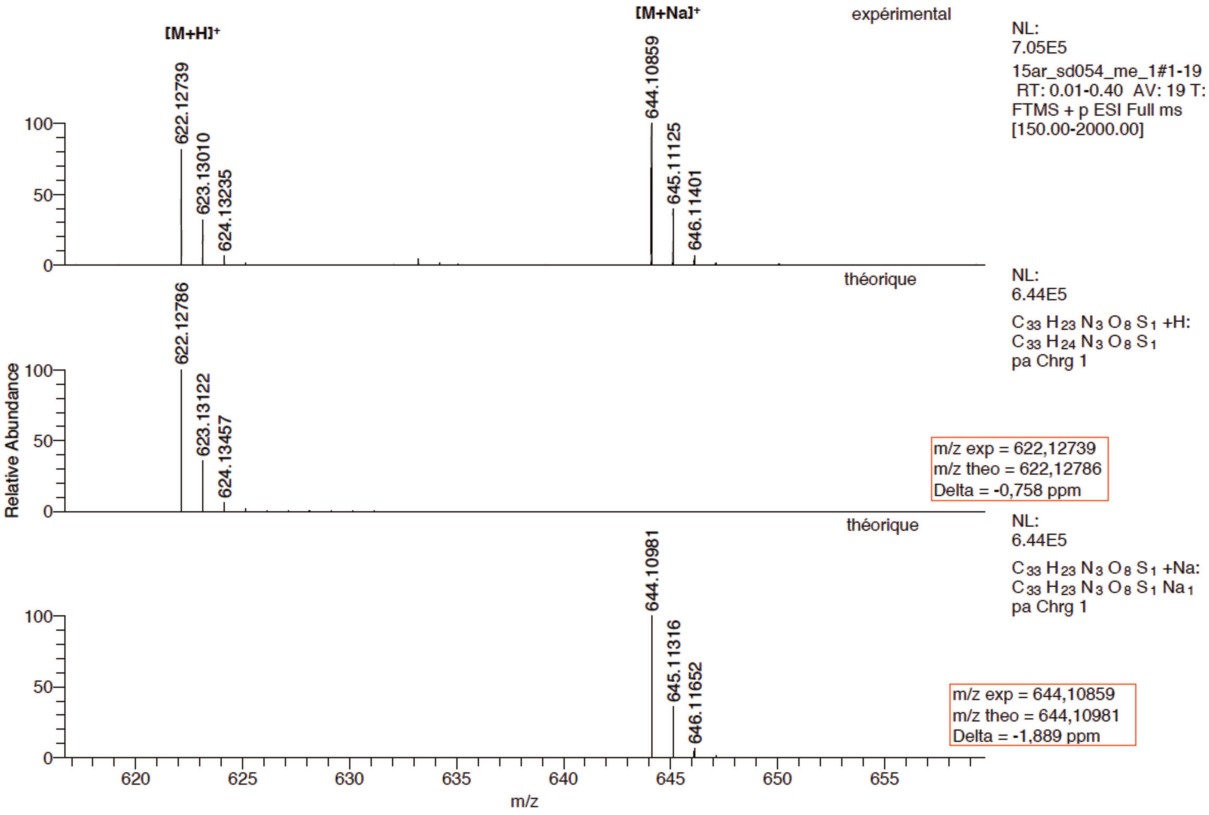
RP-HPLC elution profile (system A) of compound 5 at 260 nm



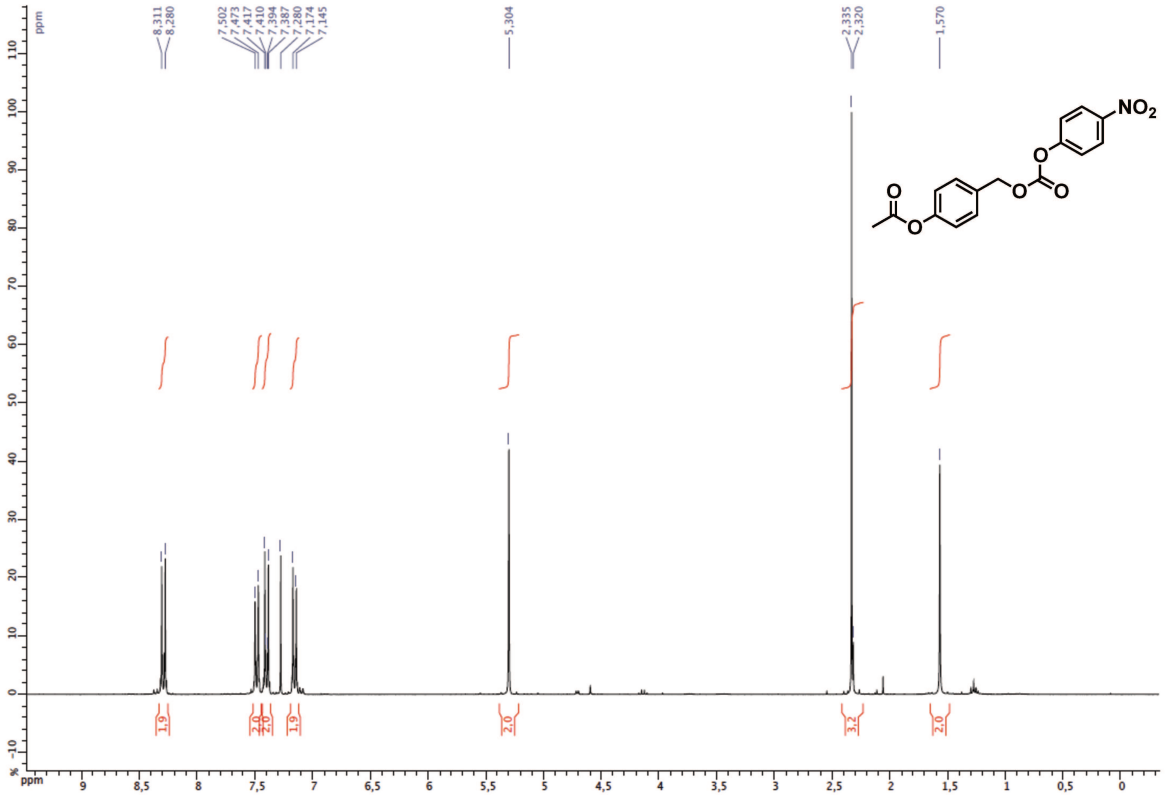
**peak found in mobile phase*

ESI+ mass spectrum (high resolution) of compound 5

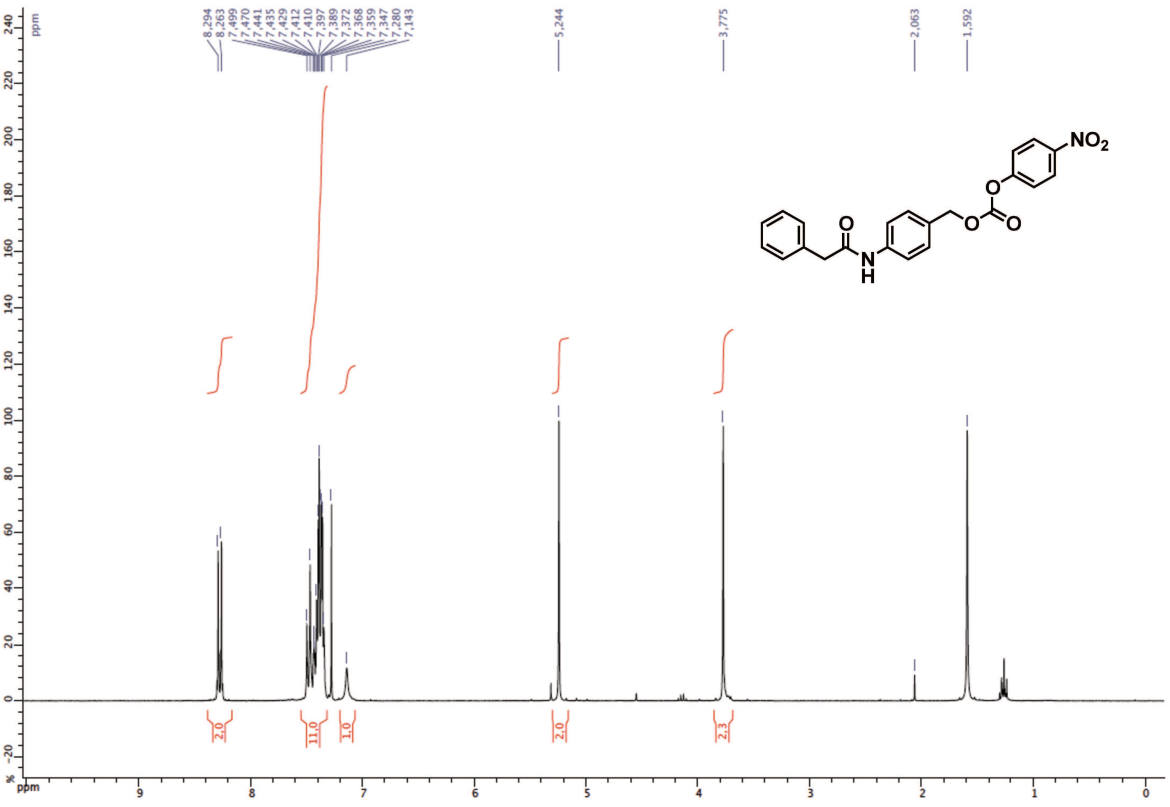




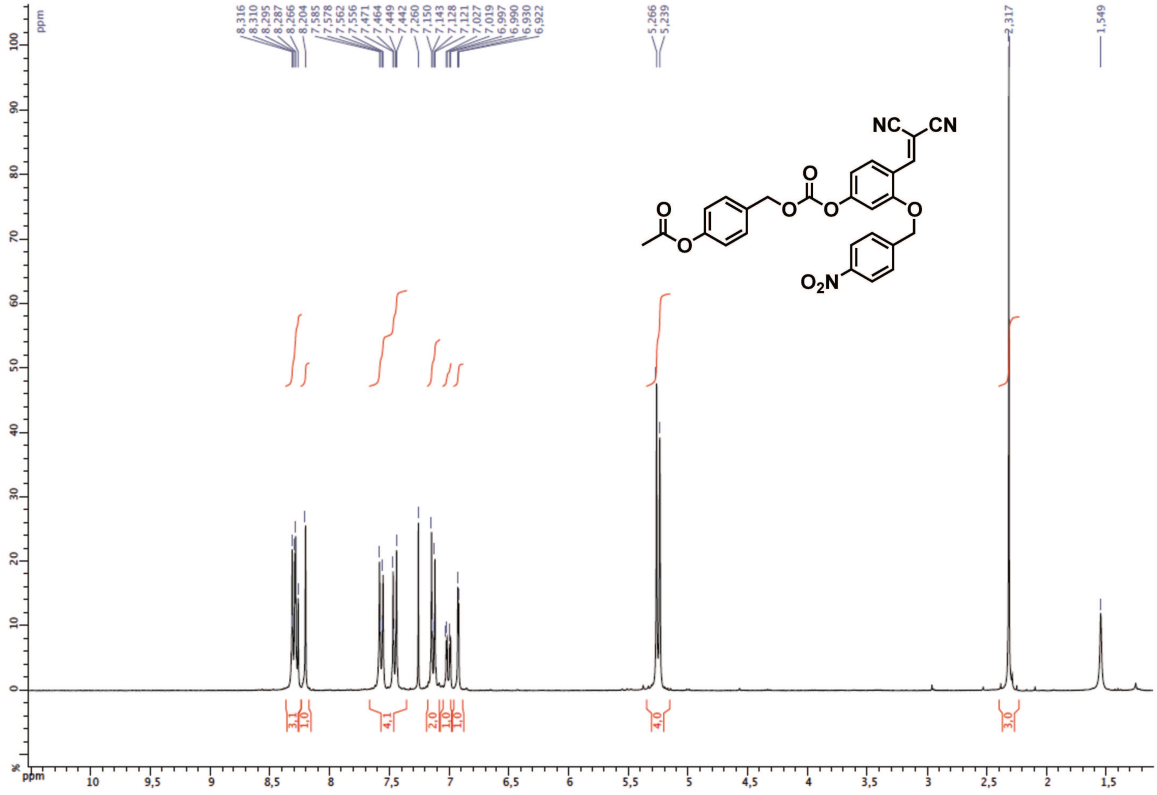
^{1}H NMR spectrum of compound 6 recorded in $CDCl_3$ at 300 MHz



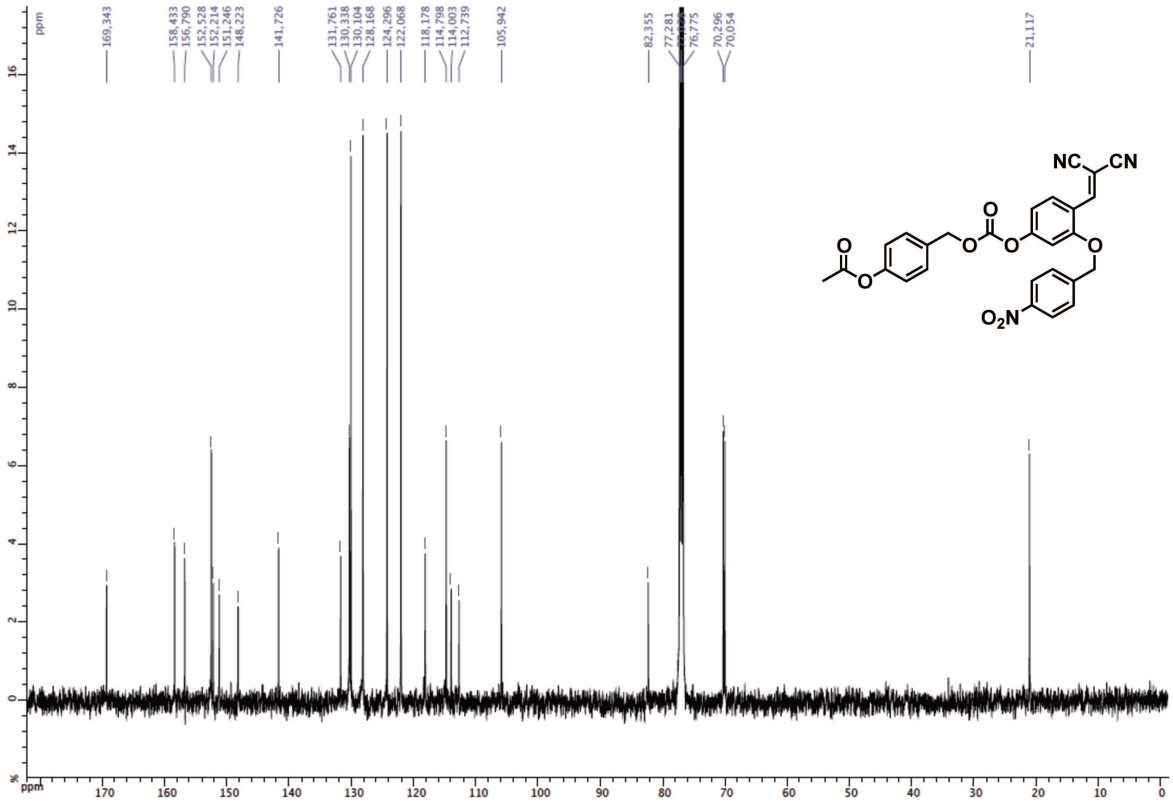
^{1}H NMR spectrum of compound 7 recorded in $CDCl_3$ at 300 MHz



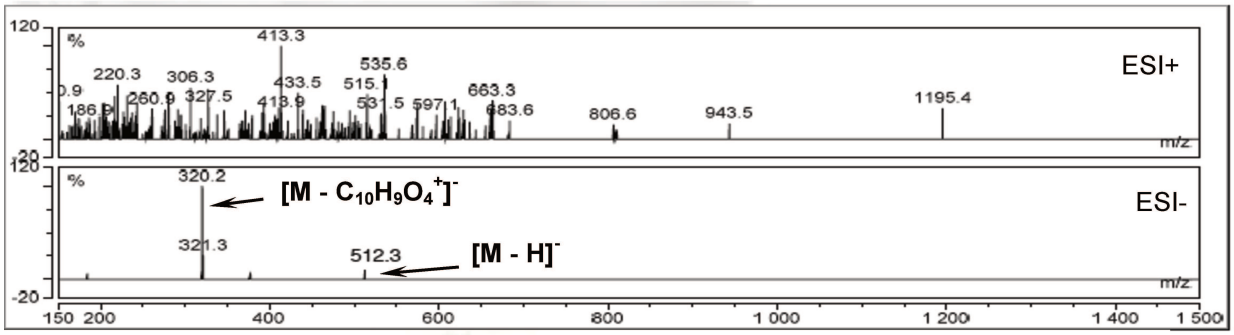
^{1}H NMR spectrum of compound 8 recorded in $CDCl_3$ at 300 MHz

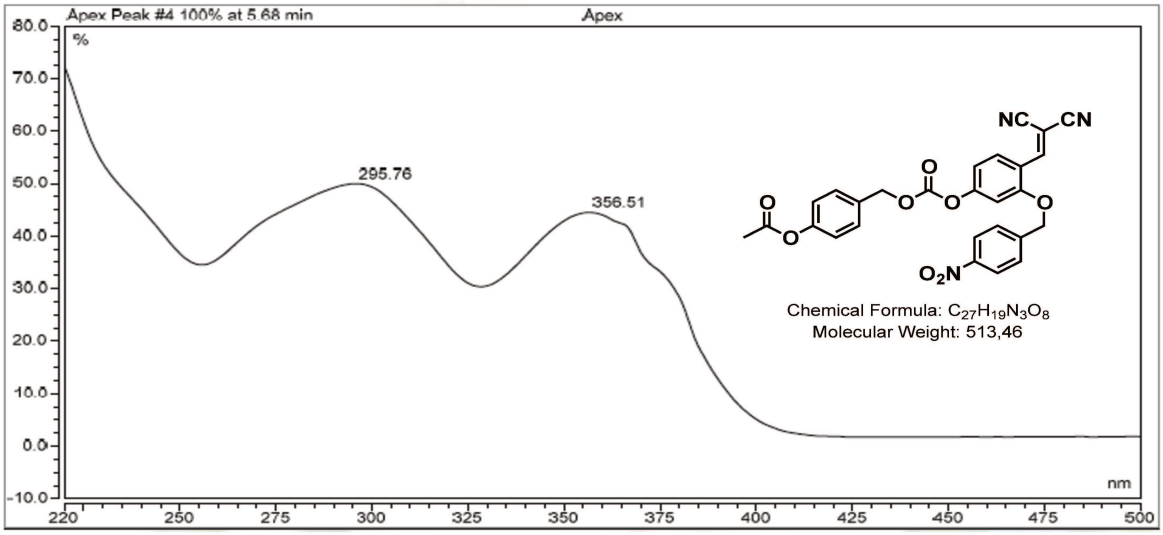


^{13}C NMR spectrum of compound 8 recorded in $CDCl_3$ at 125 MHz

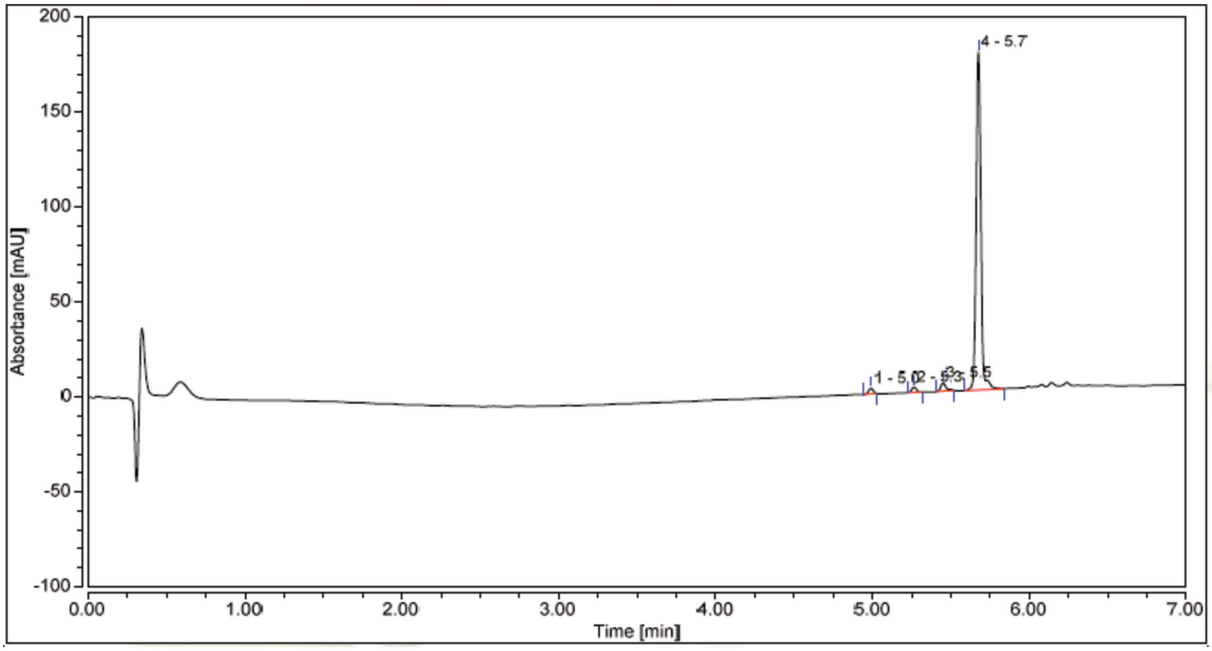


ESI-/ESI+ mass spectrum (low resolution) and UV-vis spectrum of compound 8

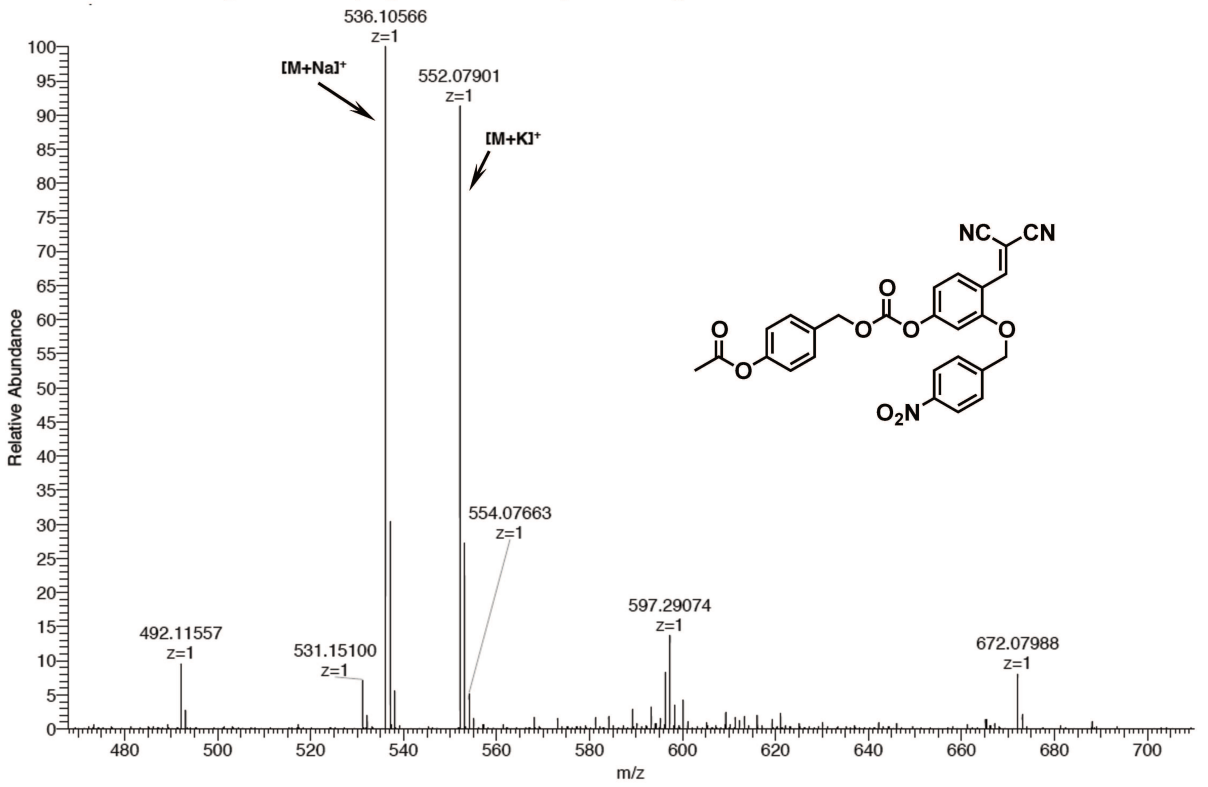


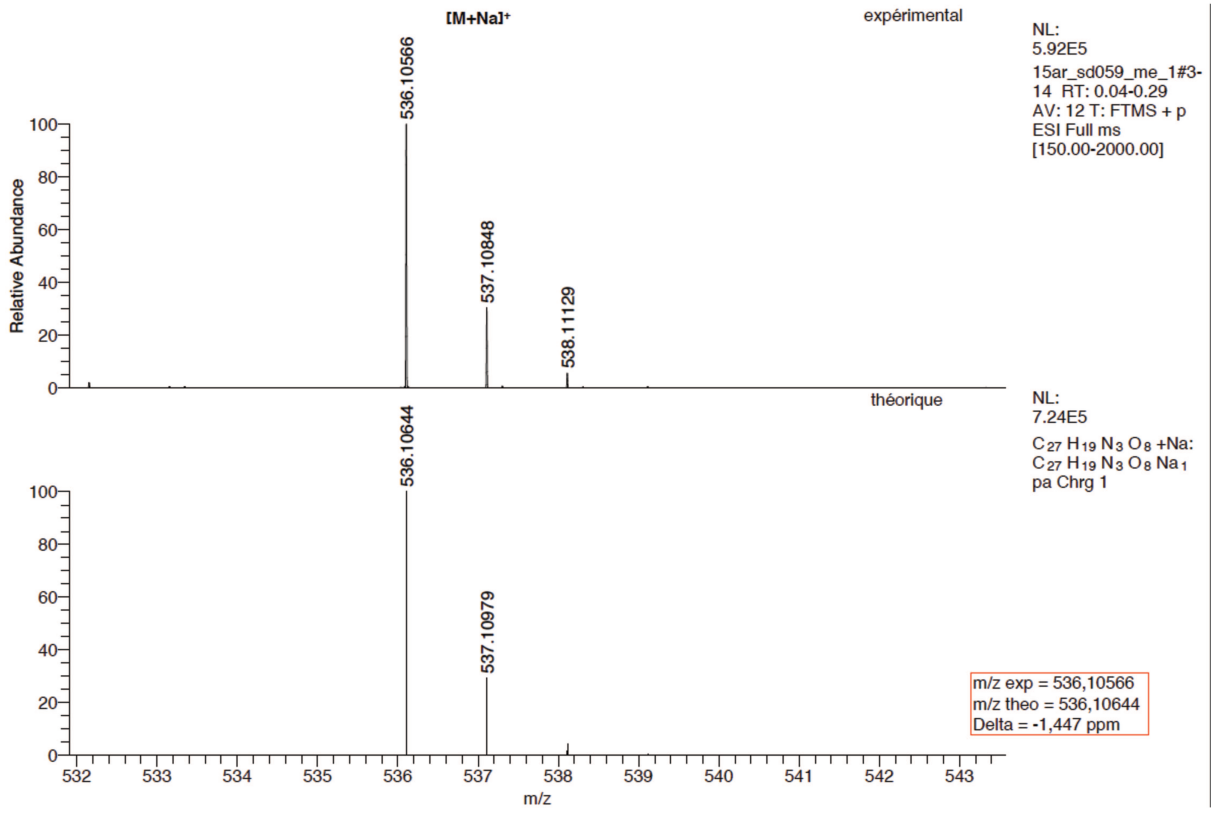


RP-HPLC elution profile (system A) of compound 8 at 260 nm

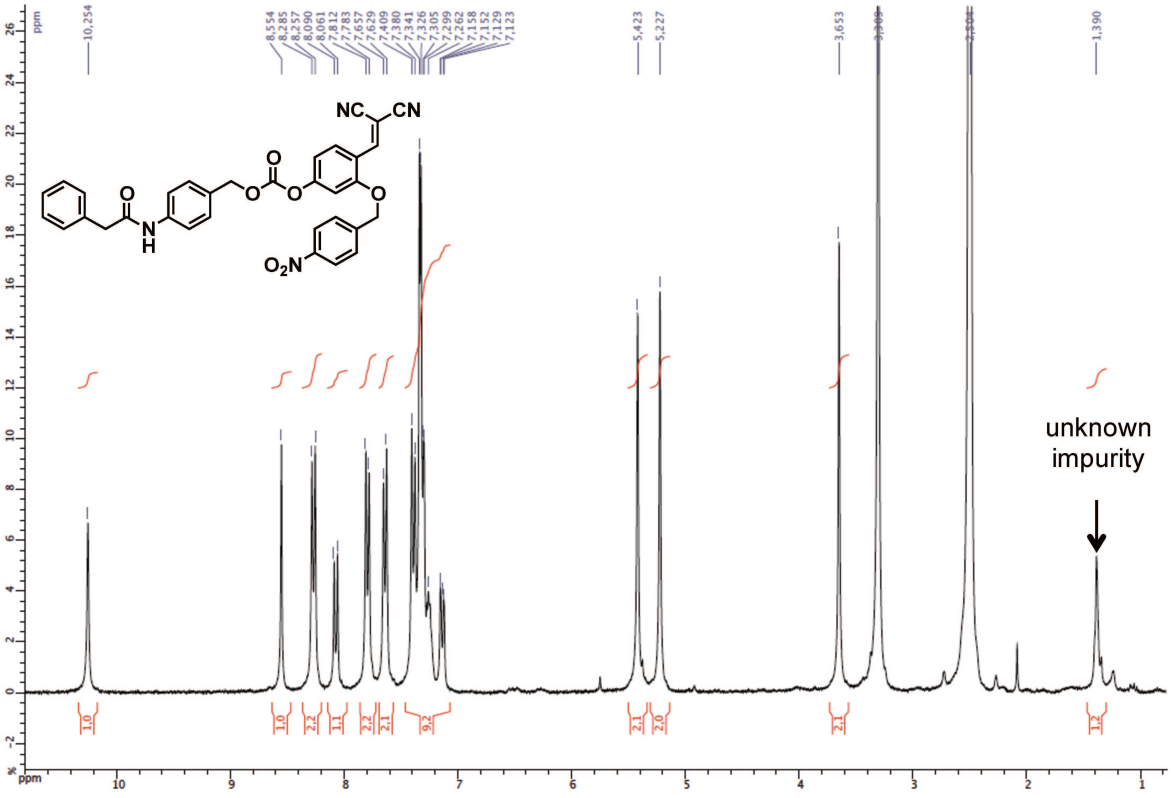


ESI+ mass spectrum (high resolution) of compound 8

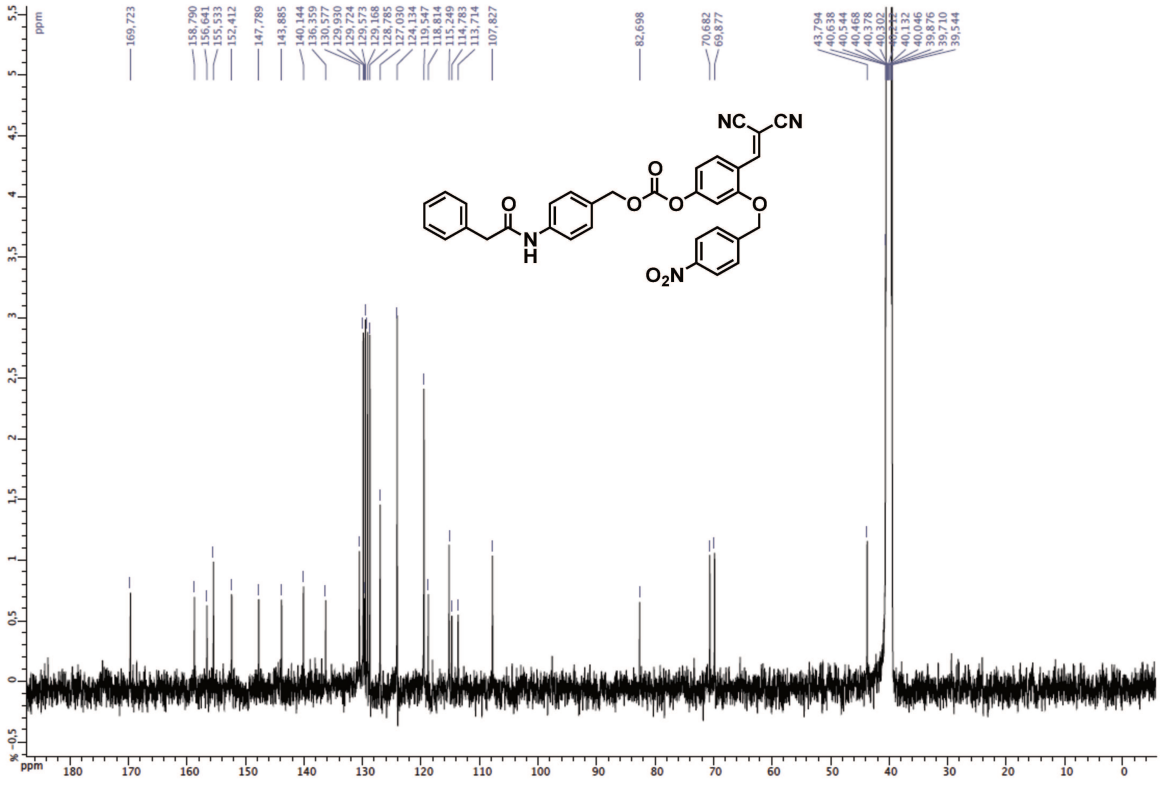




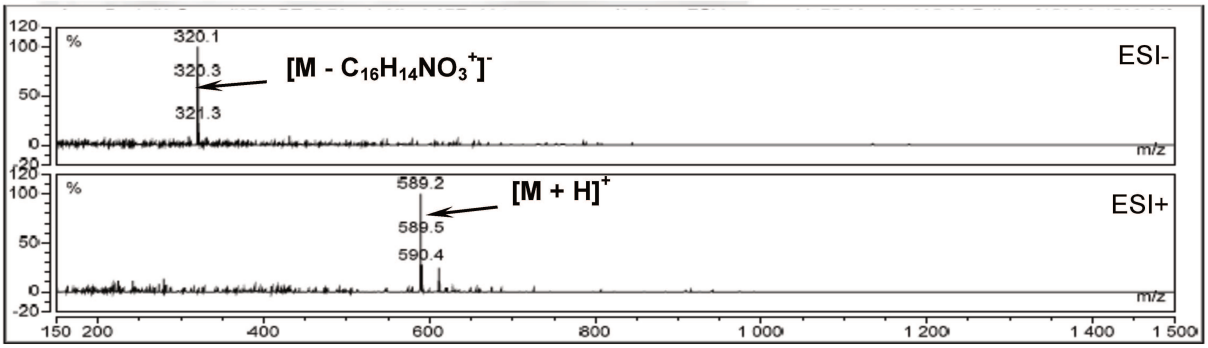
^{1}H NMR spectrum of compound 9 recorded in DMSO-d_6 at 300 MHz

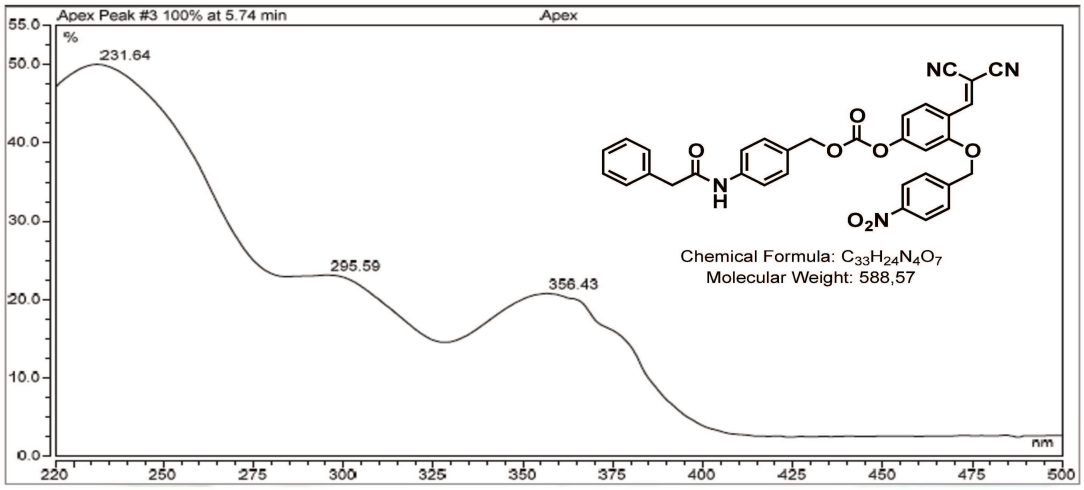


^{13}C NMR spectrum of compound 9 recorded in DMSO-d_6 at 125 MHz

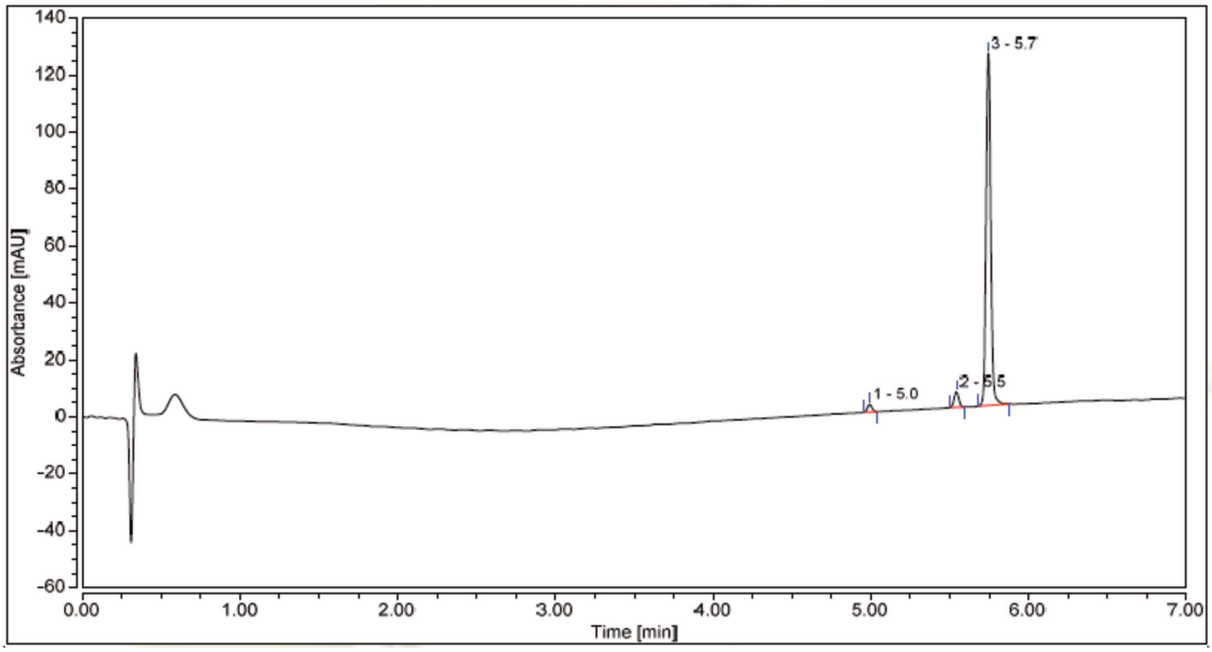


ESI-/ESI+ mass spectrum (low resolution) and UV-vis spectrum of compound 9





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



ESI+ mass spectrum (high resolution) of compound 9

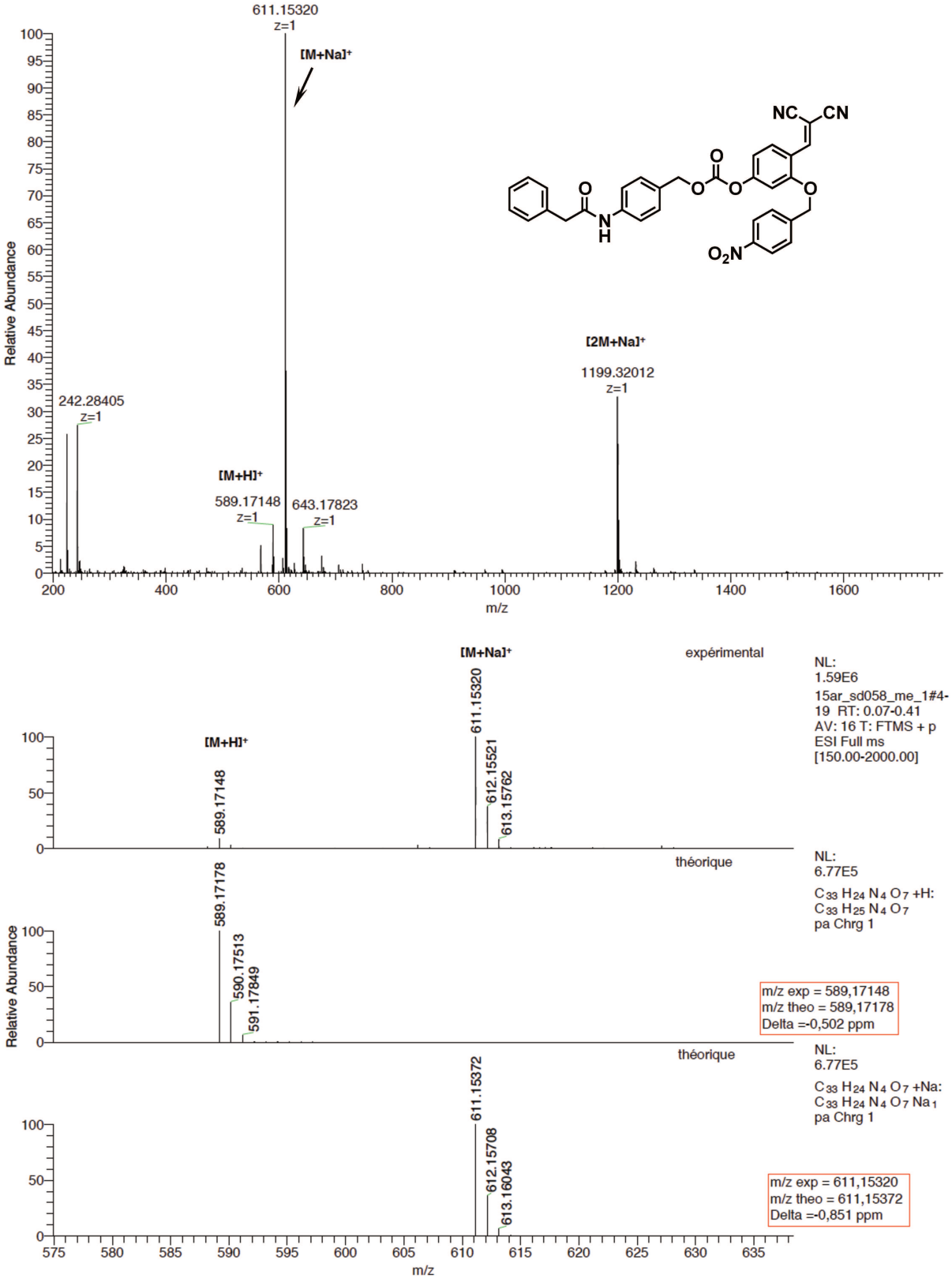


Fig S1. Normalised absorption spectra of fluorogenic probes 5, 8 and 9 in PB (+ 0.3% DMSO) at 25 °C

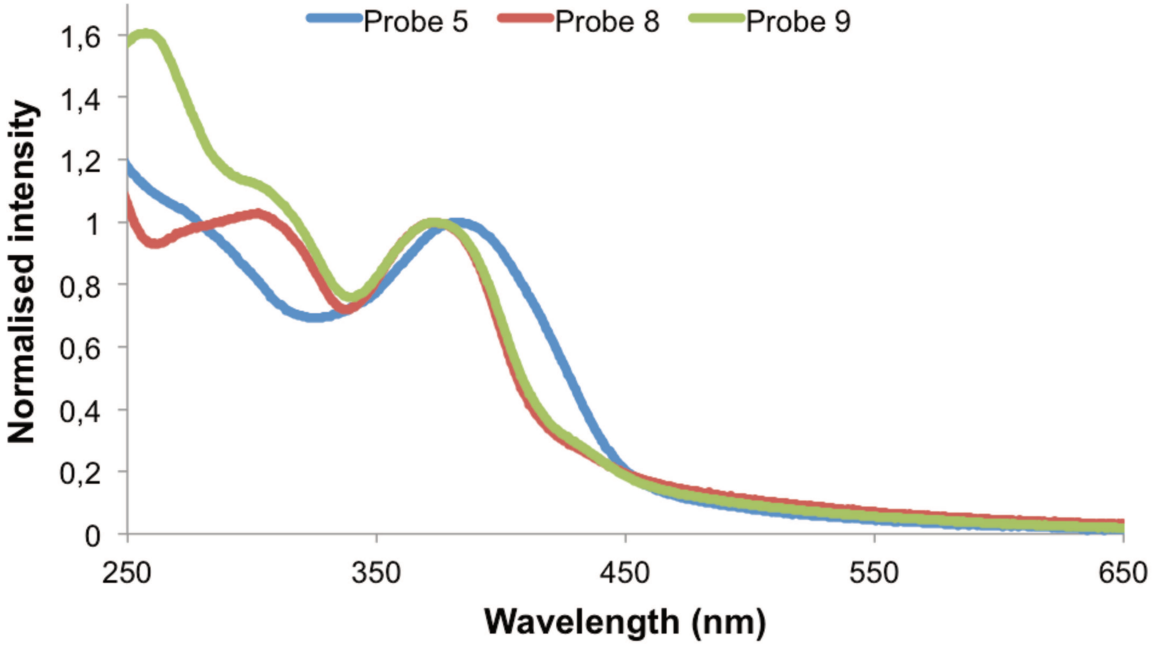


Fig S2. Normalised absorption, excitation (Em. 510 nm) and emission (Ex. 400 nm) spectra of 3-cyano-7-hydroxy-2-iminocoumarin in PB at 25 °C.

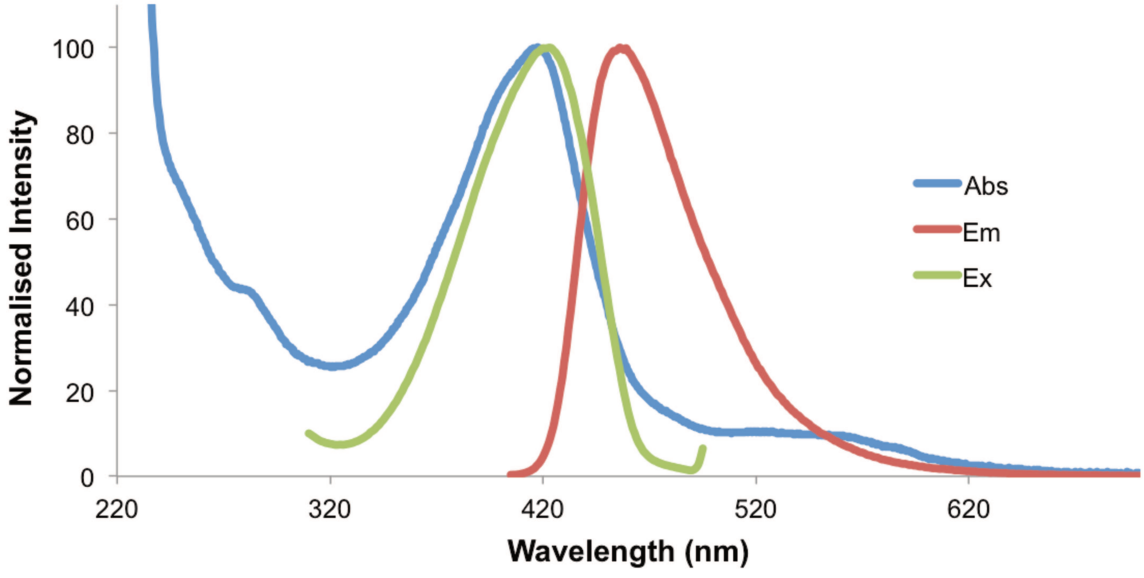


Fig S3. Normalised absorption, excitation (Em. 530 nm) and emission (Ex. 370 nm) spectra of 3-cyano-7-hydroxycoumarin in PB at 25 °C.

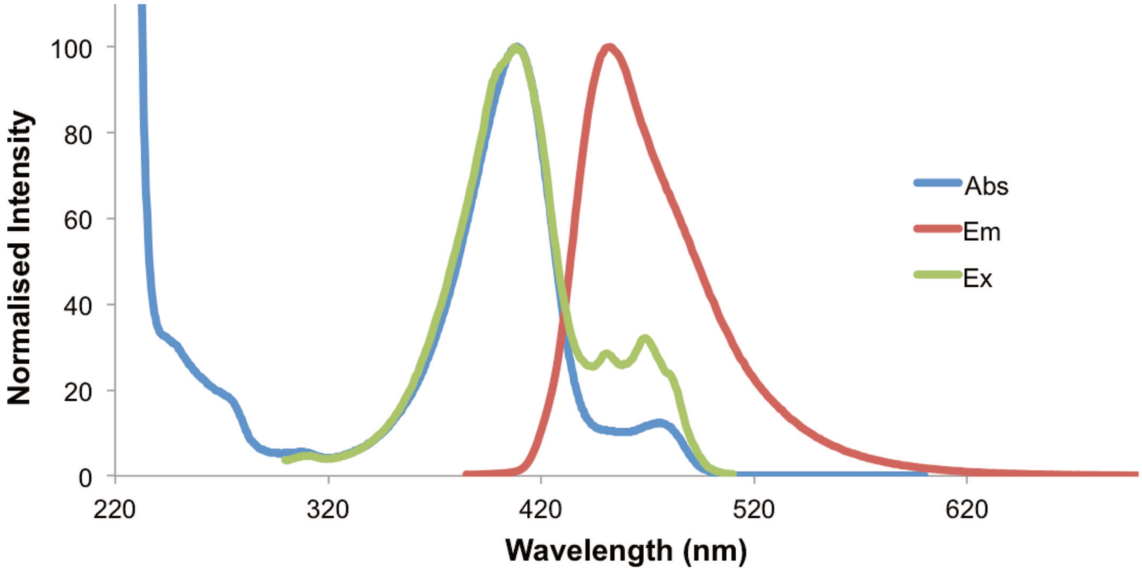
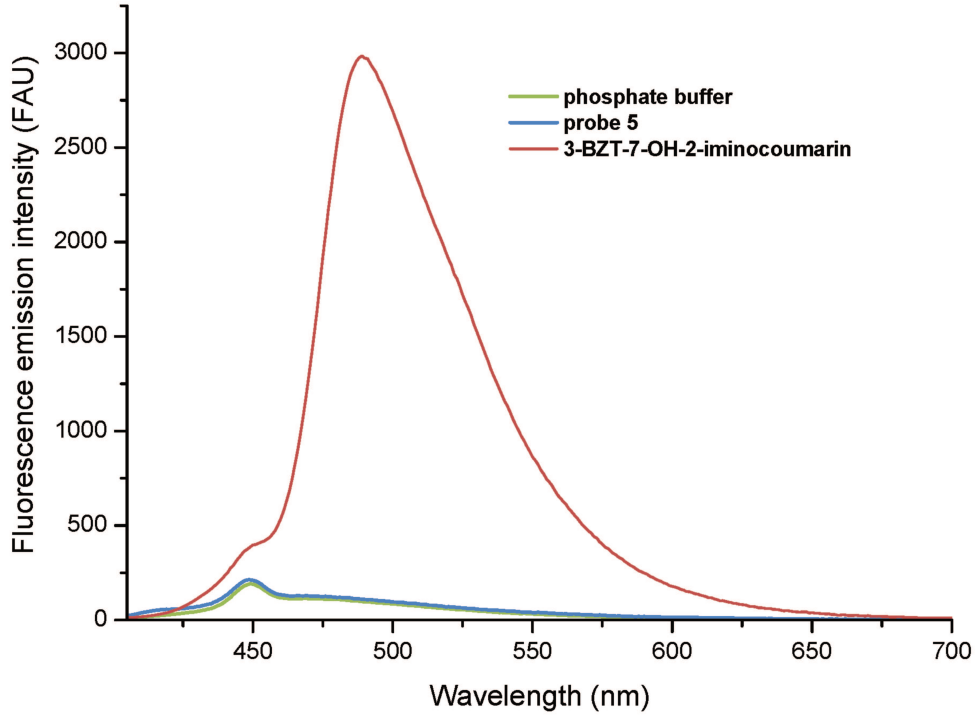
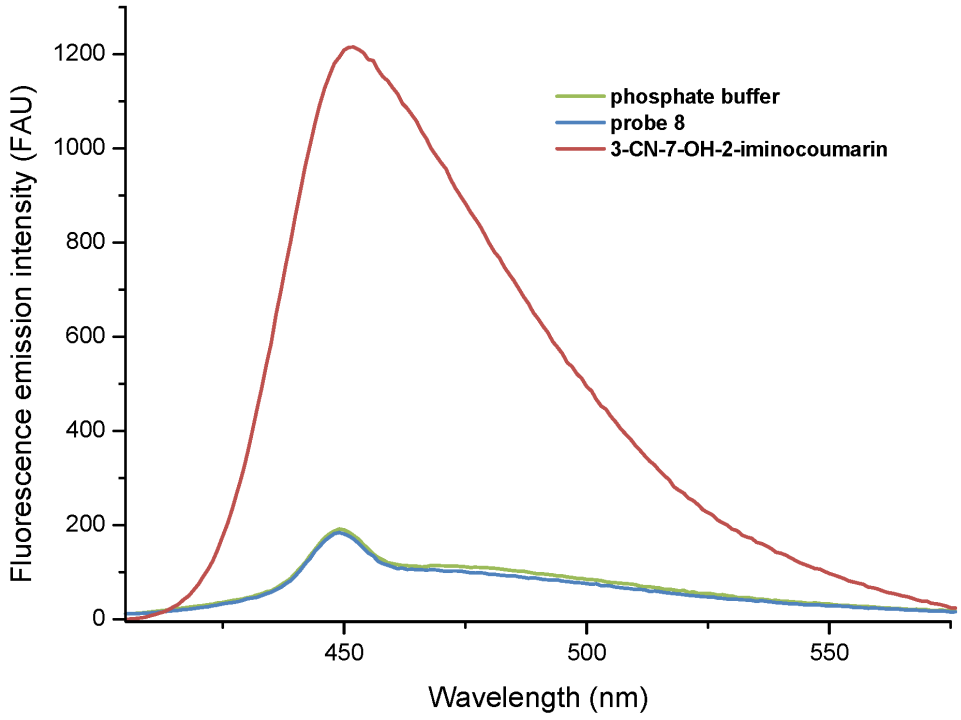


Fig S4. Overlayed fluorescence emission spectra (Ex. 390 nm) of PLE-NTR fluorogenic probe 5 and 3-(2-benzothiazolyl)-7-hydroxy-2-iminocoumarin in PB at 25 °C (concentration: 0.1 μM)[a]



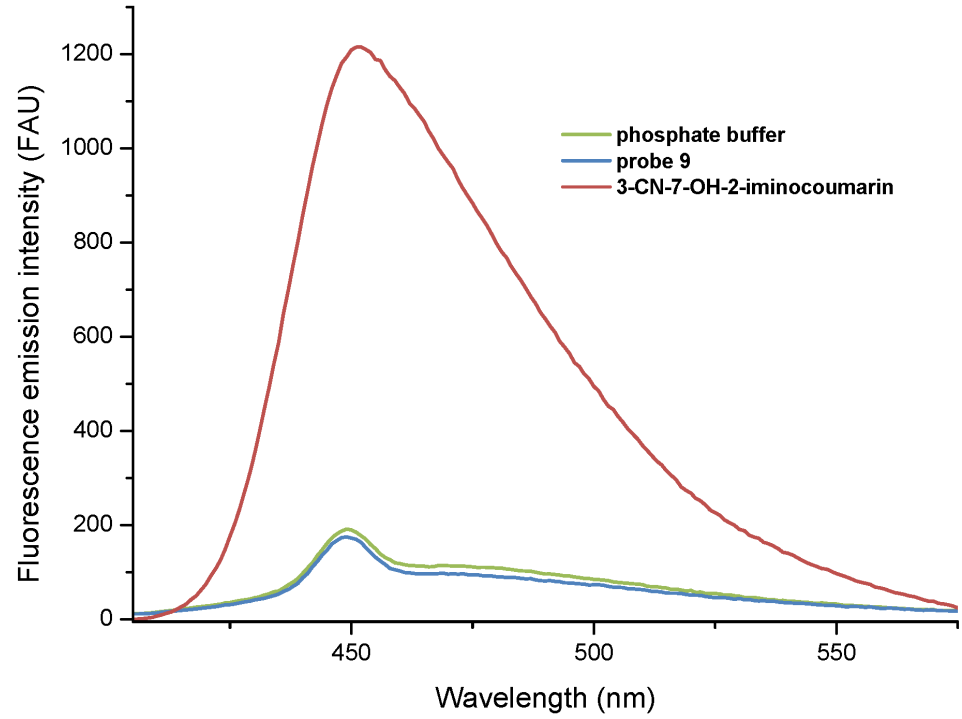
[a]*Raman scatter of water at 450 nm*

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]



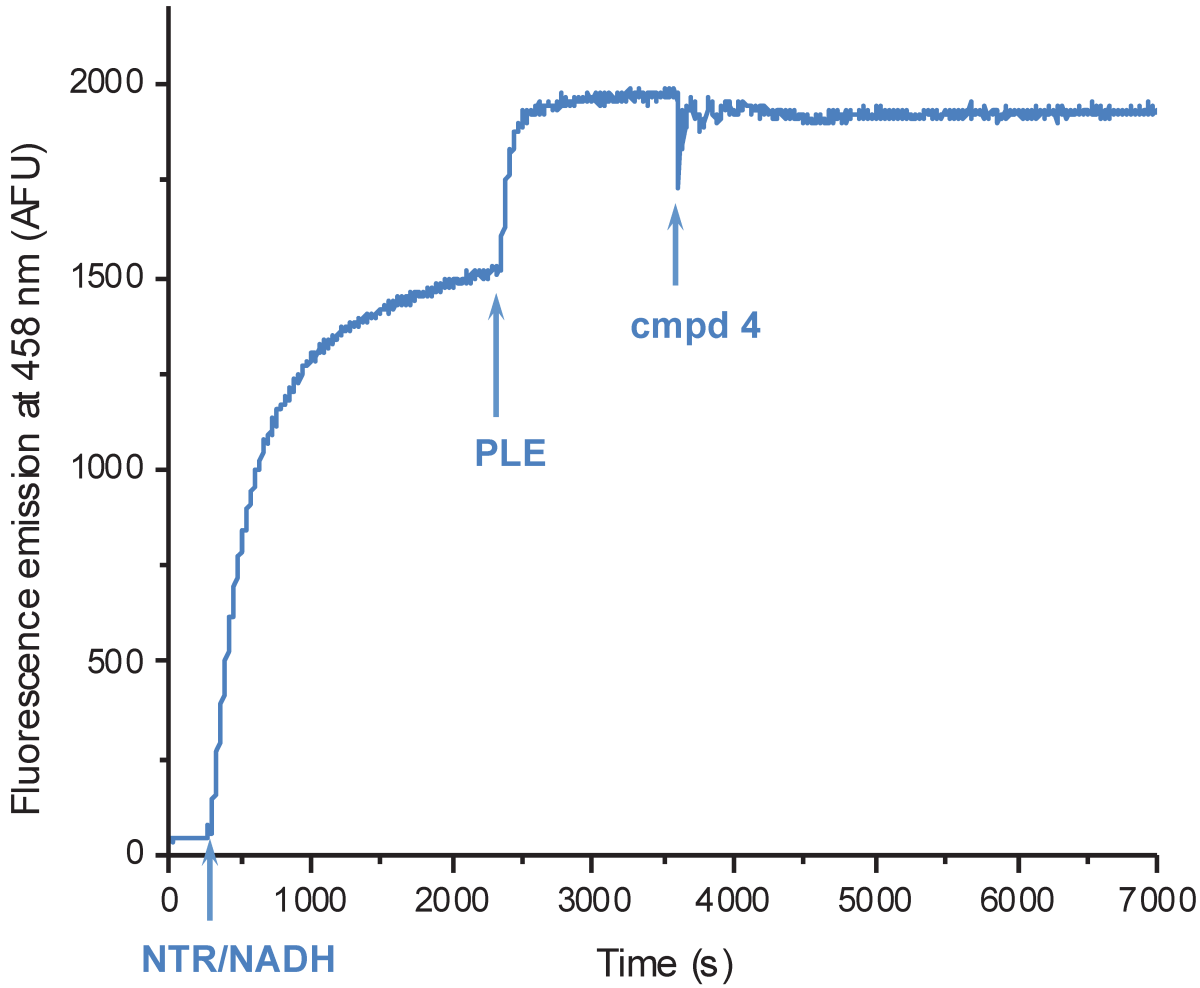
[a]*Raman scatter of water at 450 nm*

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

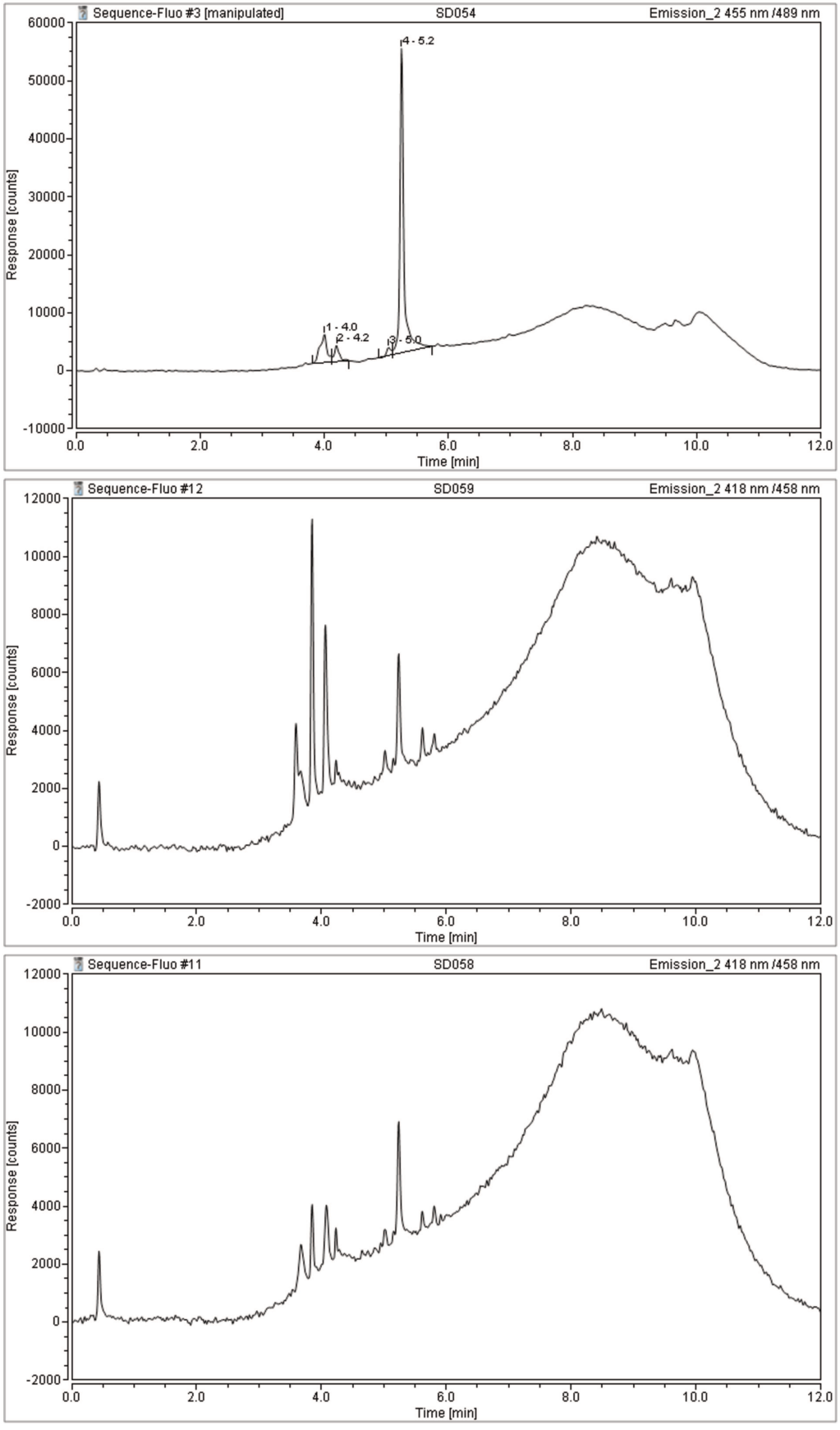
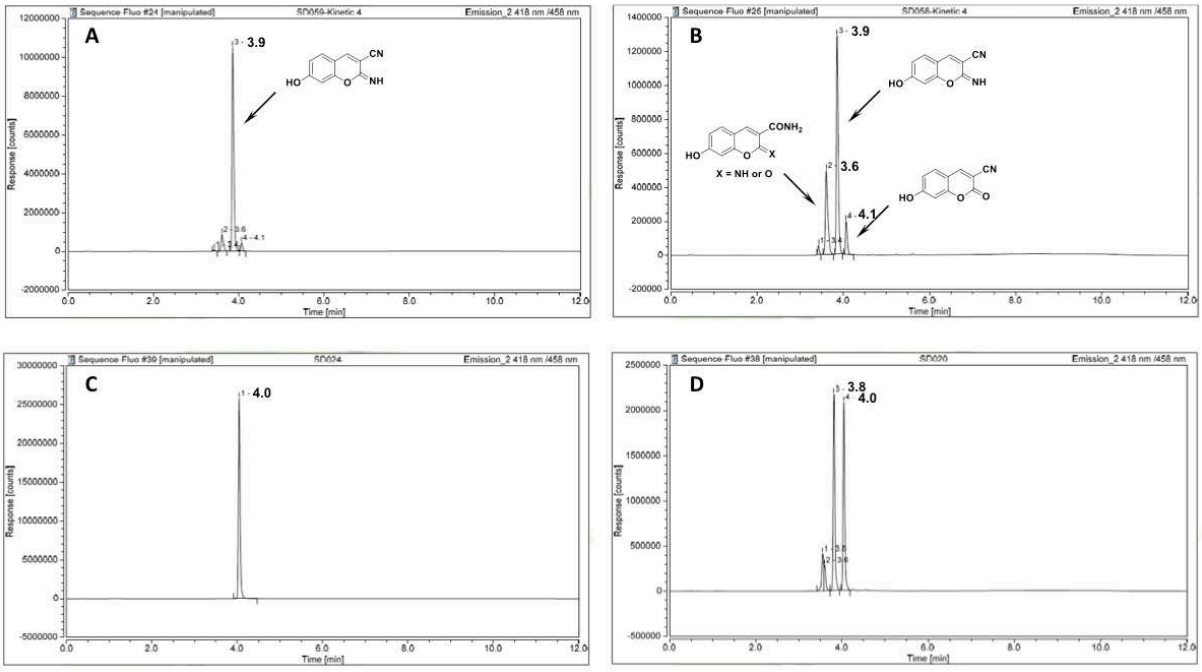
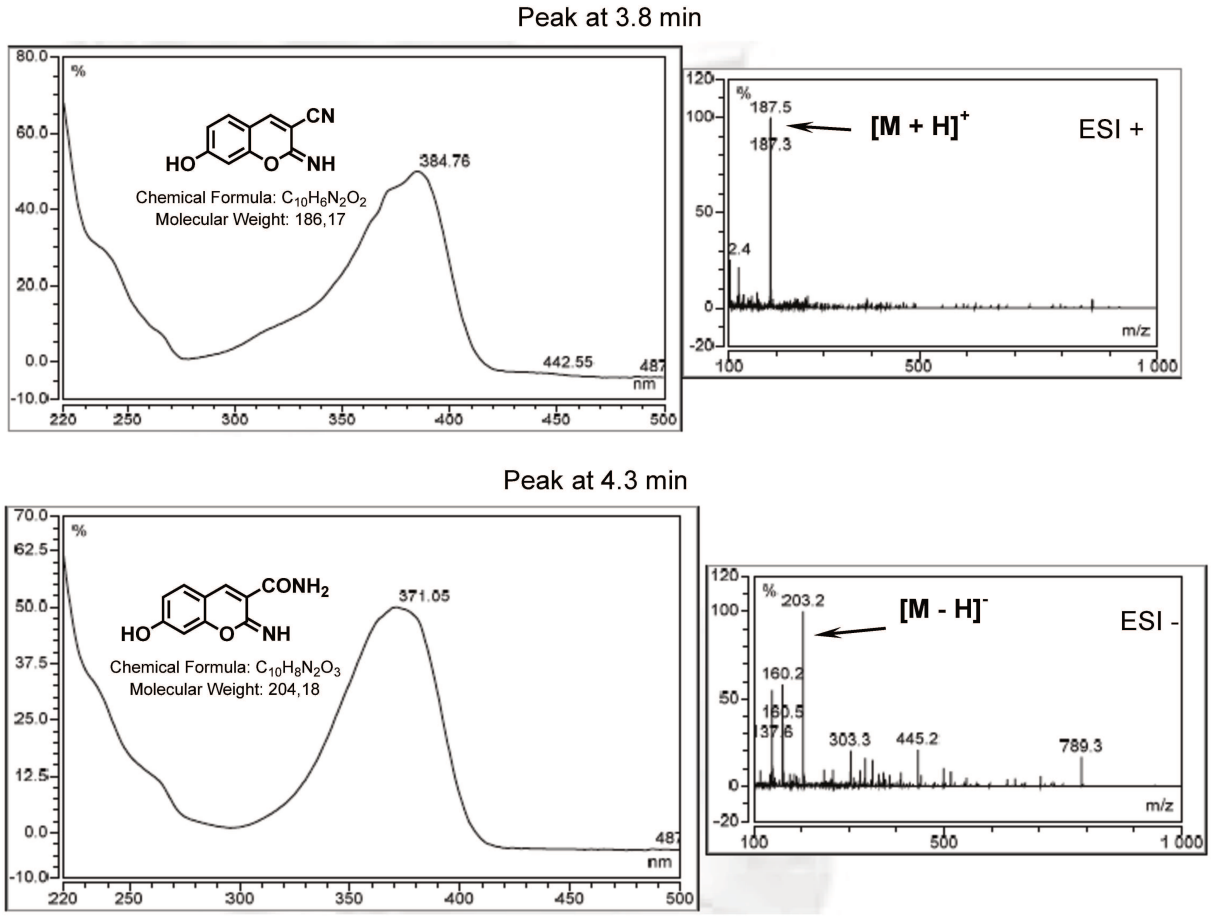


Fig S9. RP-HPLC elution profiles (fluorescence detection, system D) of enzymatic reaction mixture of cyano-based probes 8 and 9 incubated simultaneously with both enzymes: hydrolase (PLE or PGA) and NTR/NADH

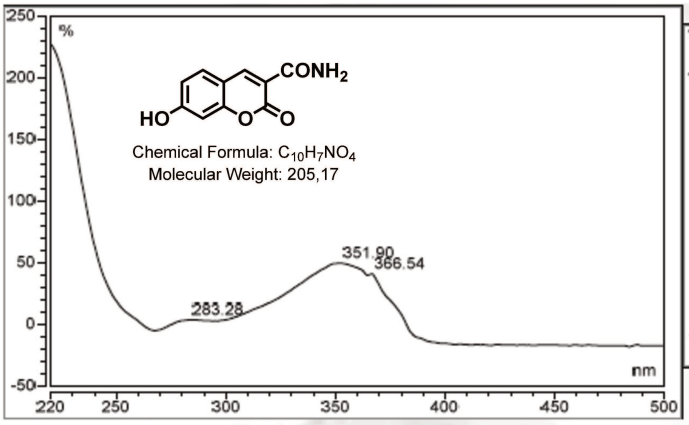


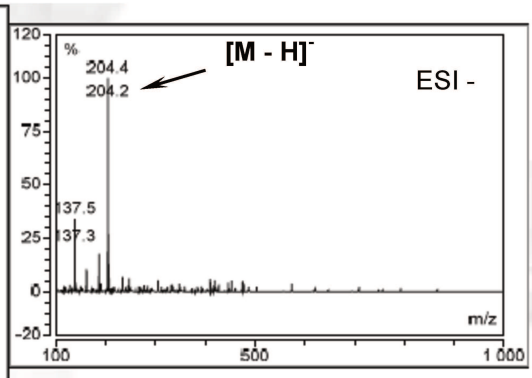
Enzymatic reaction mixtures (A-B) and authentic samples of 3-cyano-7-hydroxycoumarin (C) and 3-cyano-7-hydroxy-2-iminocoumarin (D). *Please note*: partial hydrolysis of cyano and imine moieties was occurred during HPLC analysis and incubation in PB. NADH (t_R = 3.4 min) can be properly detected at a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

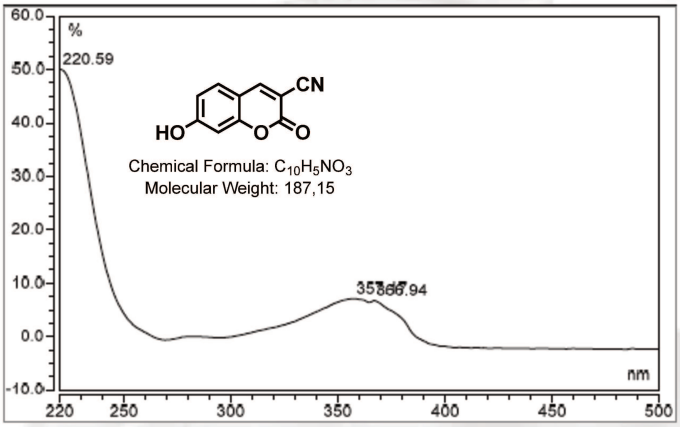


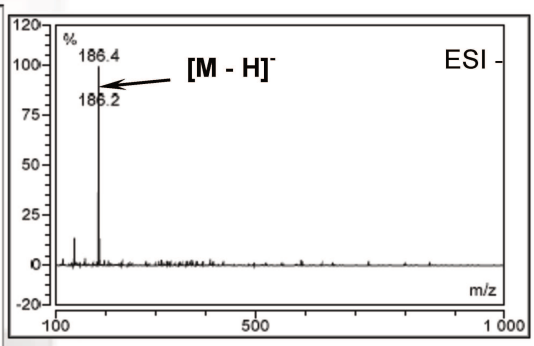
Peak at 5.5 min



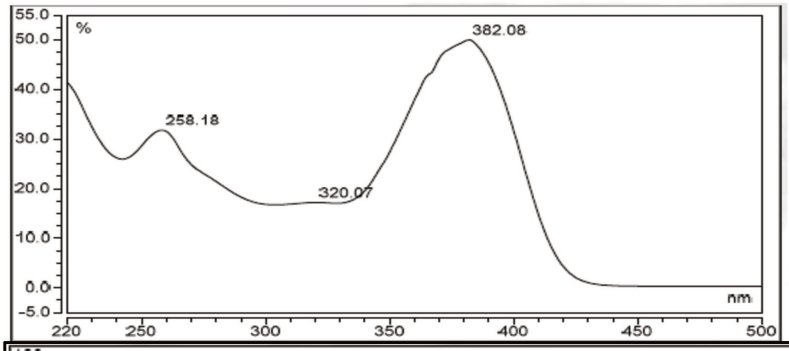


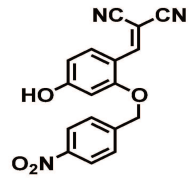
Peak at 5.9 min



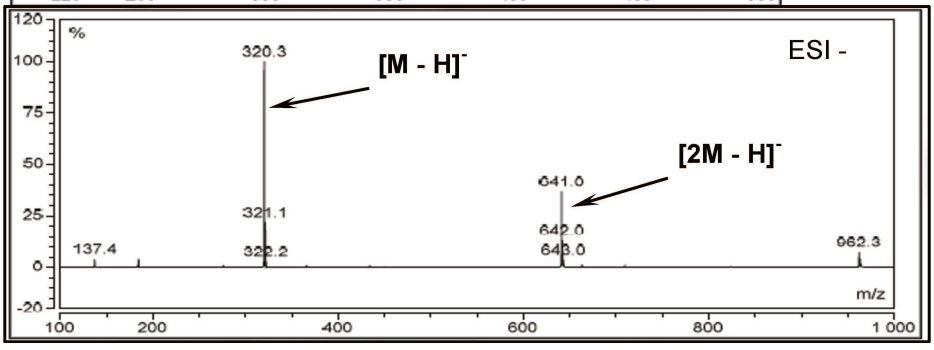


Peak at 7.7 min

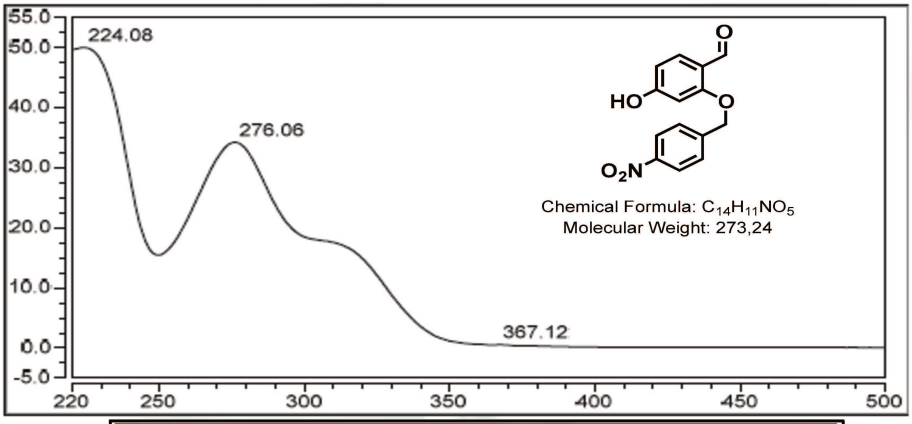


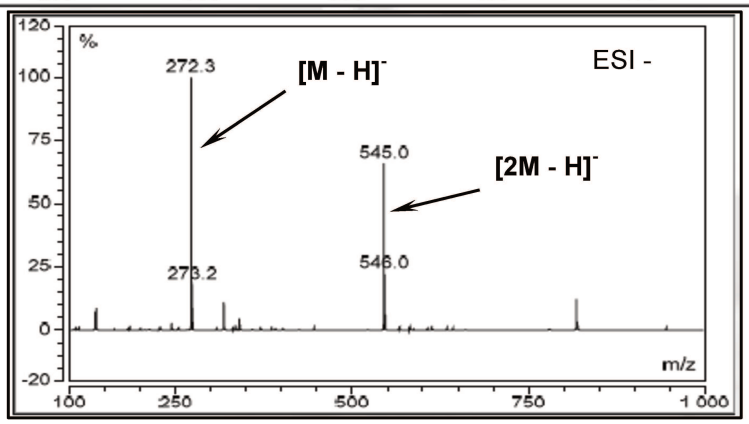


Chemical Formula: $C_{17}H_{11}N_3O_4$
Molecular Weight: 321,29

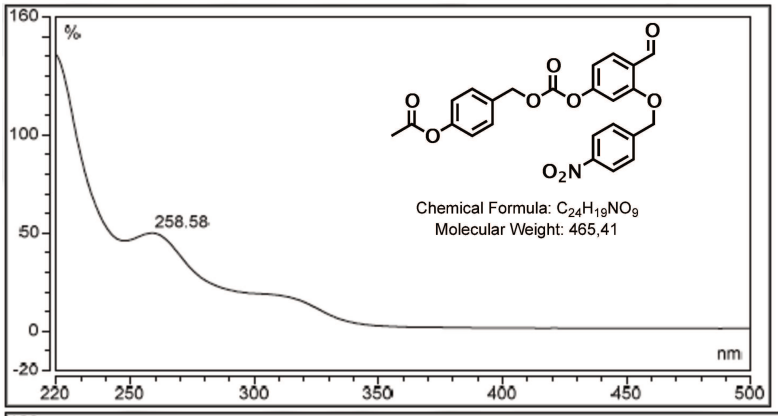


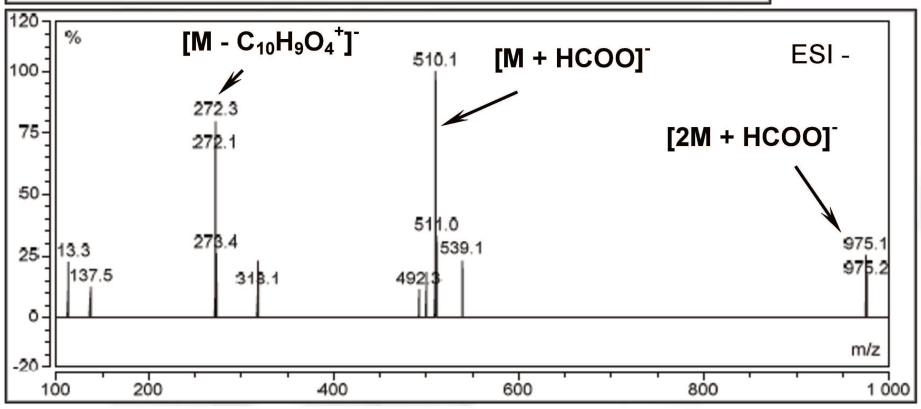
Peak at 7.0 min



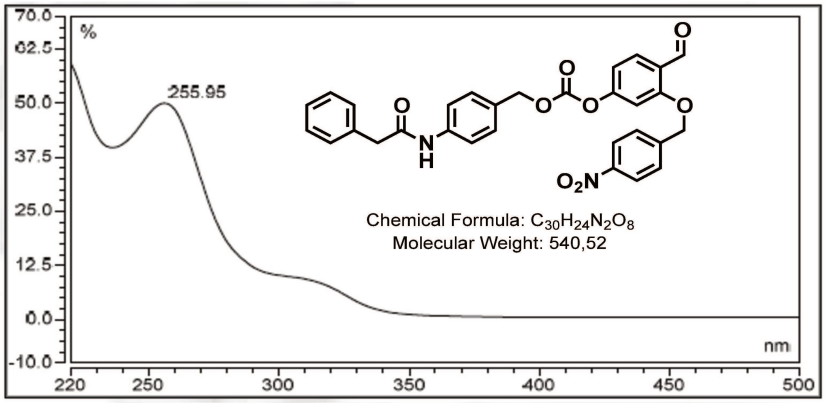


Peak at 8.18 min





Peak at 8.3 min



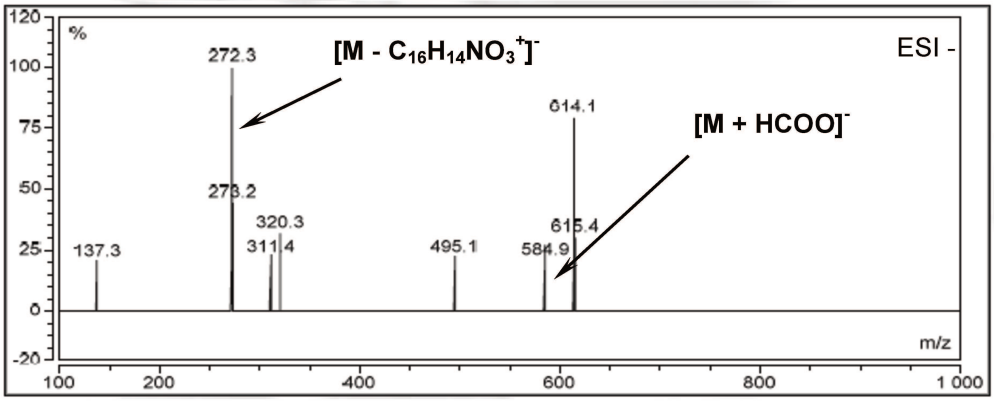
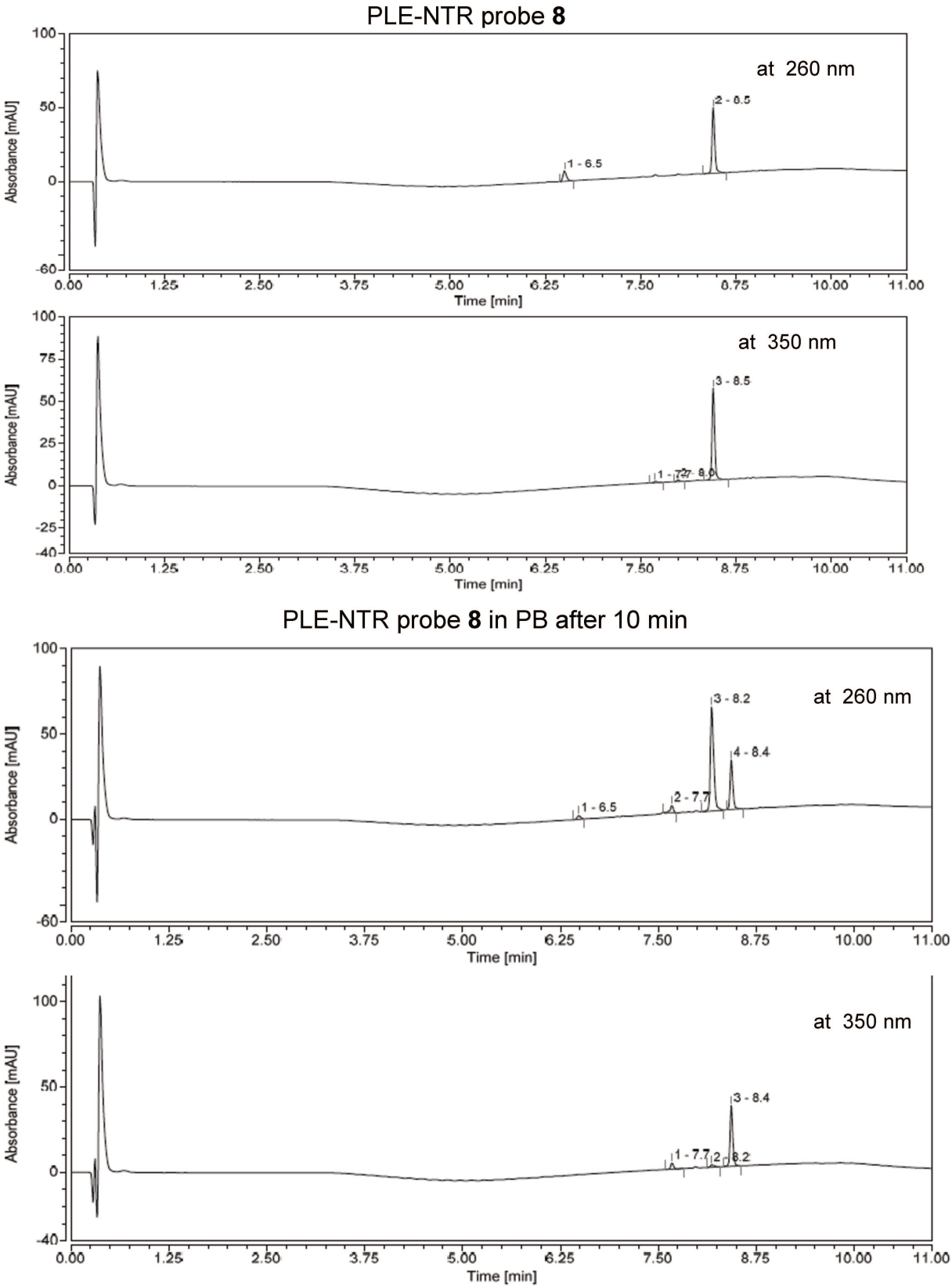
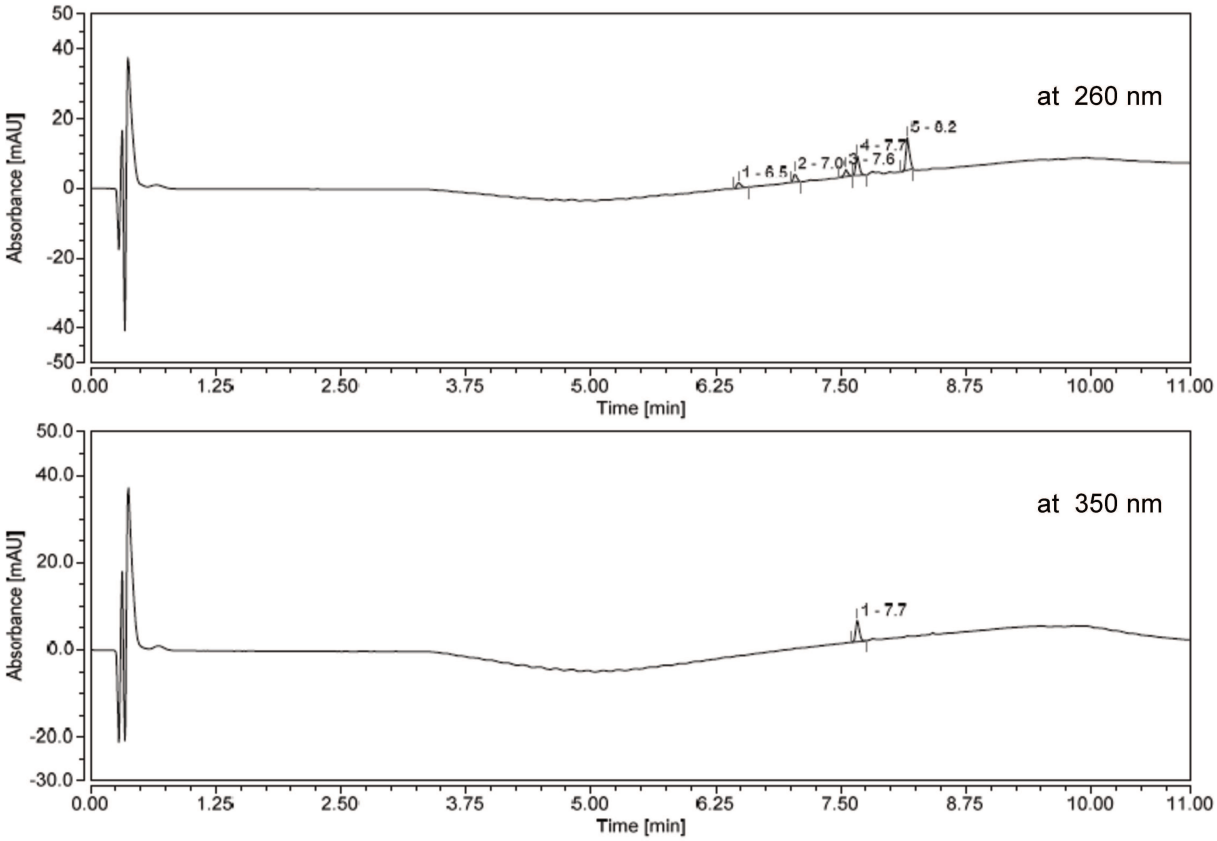


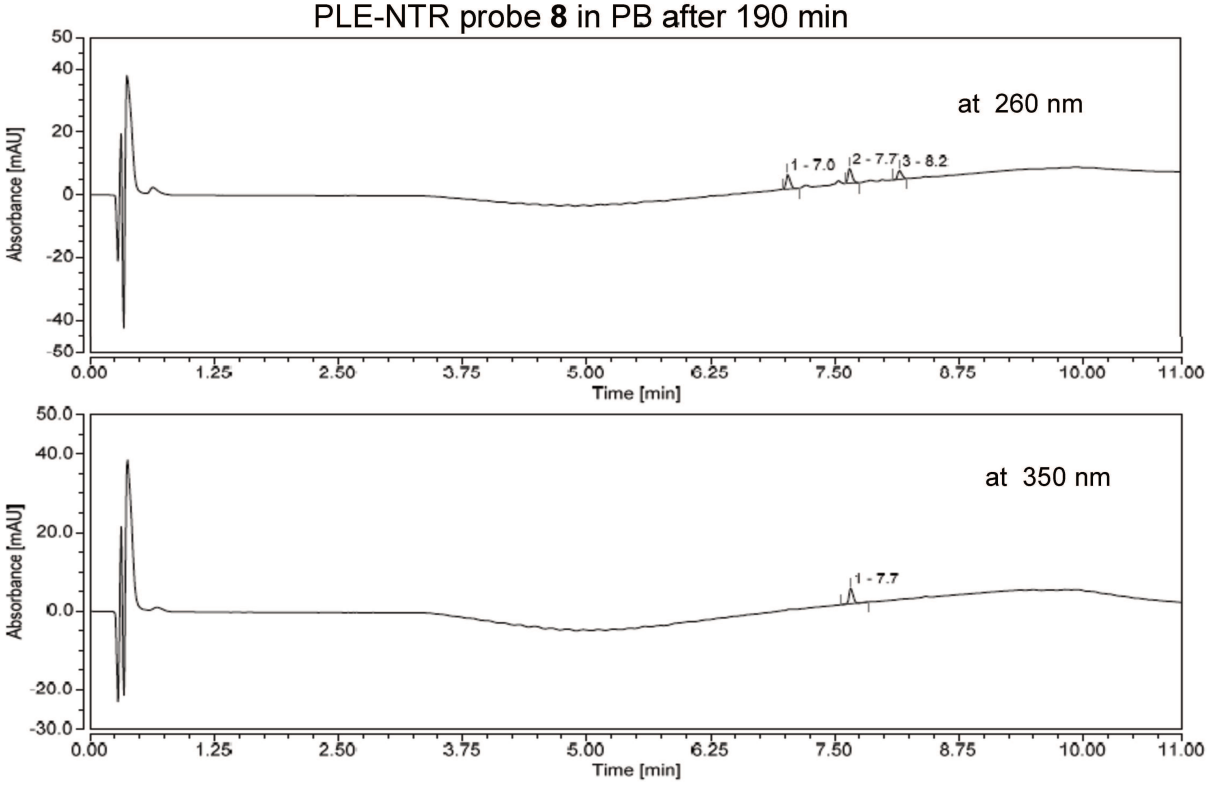
Fig S11. RP-HPLC elution profiles (system C) of fluorogenic probe 8 after incubation in PB alone or with enzymes (PLE and NTR/NADH)



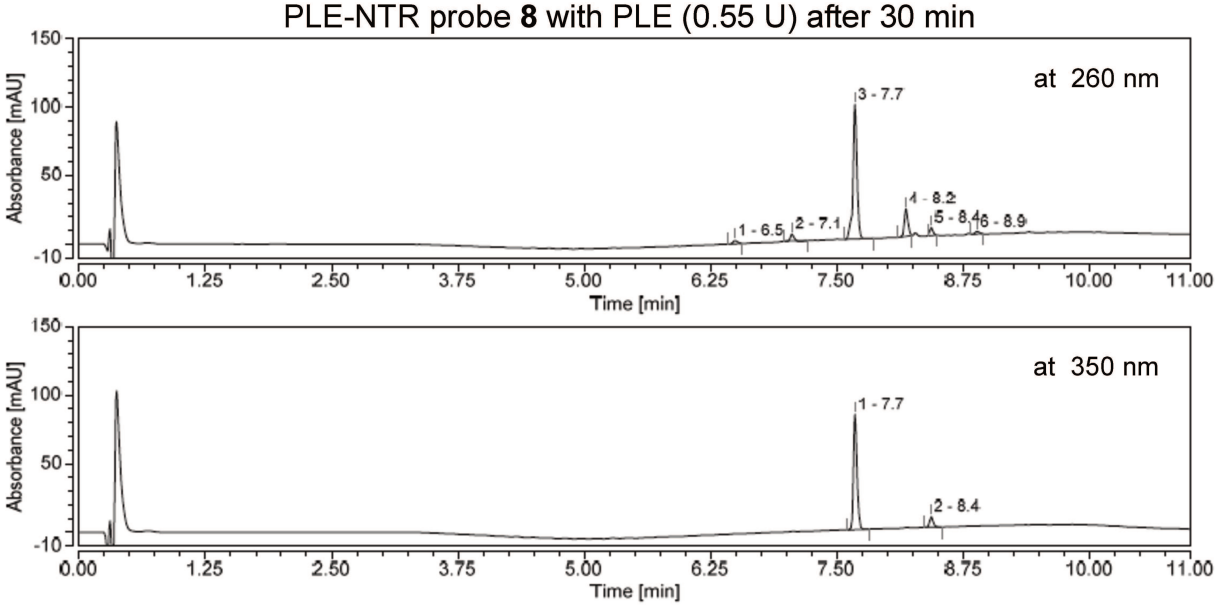
PLE-NTR probe **8** in PB after 90 min



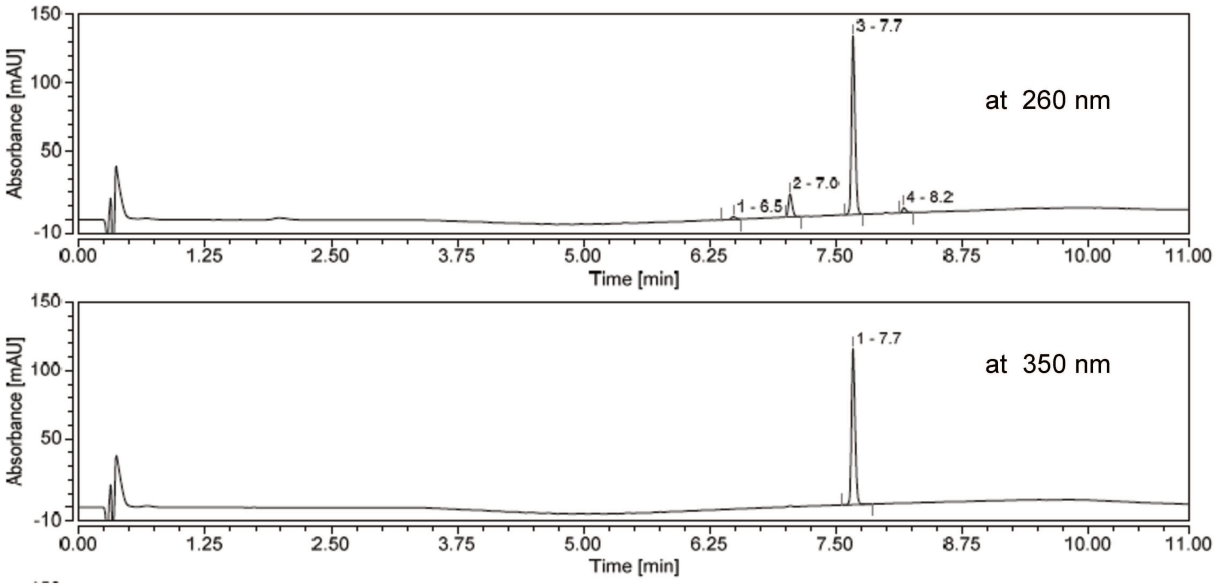
PLE-NTR probe **8** in PB after 190 min



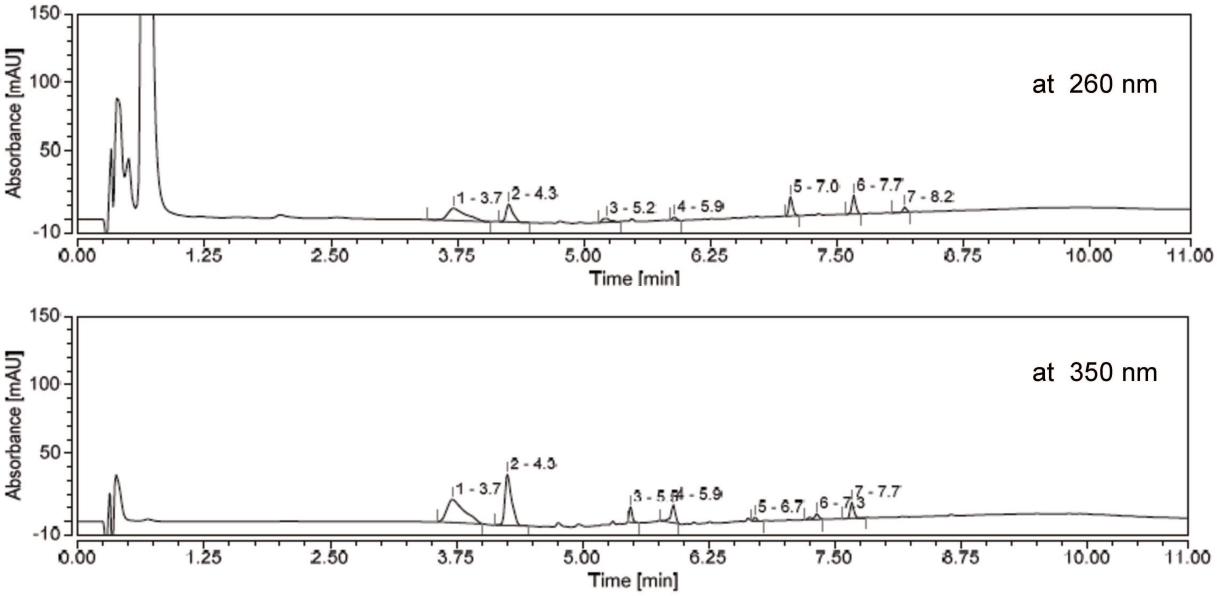
PLE-NTR probe **8** with PLE (0.55 U) after 30 min



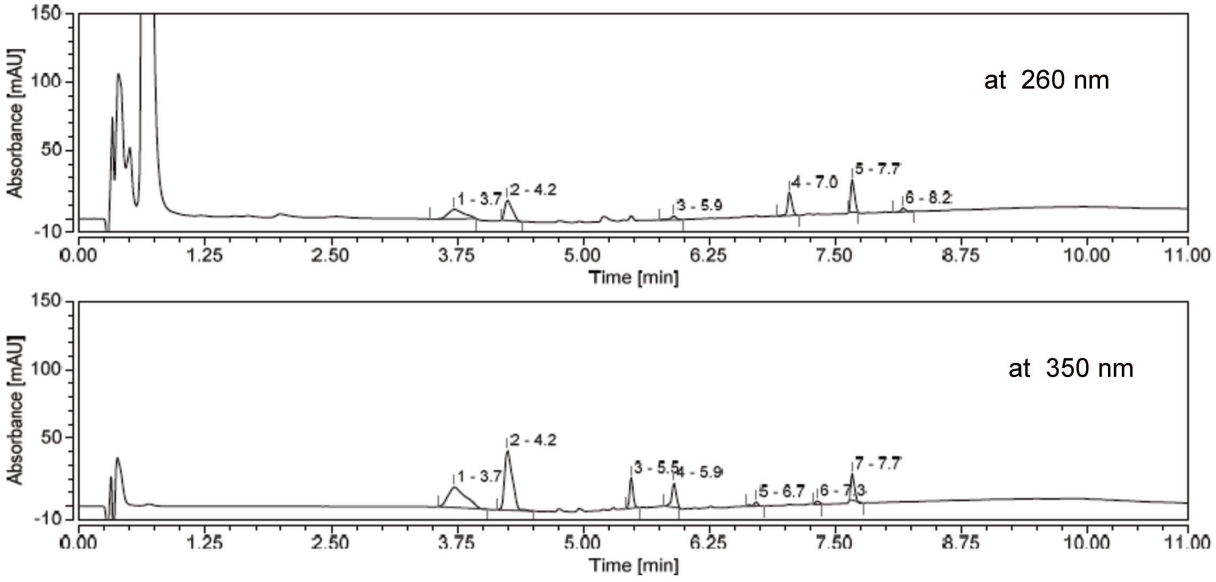
PLE-NTR probe **8** with PLE (0.55 U) after 60 min



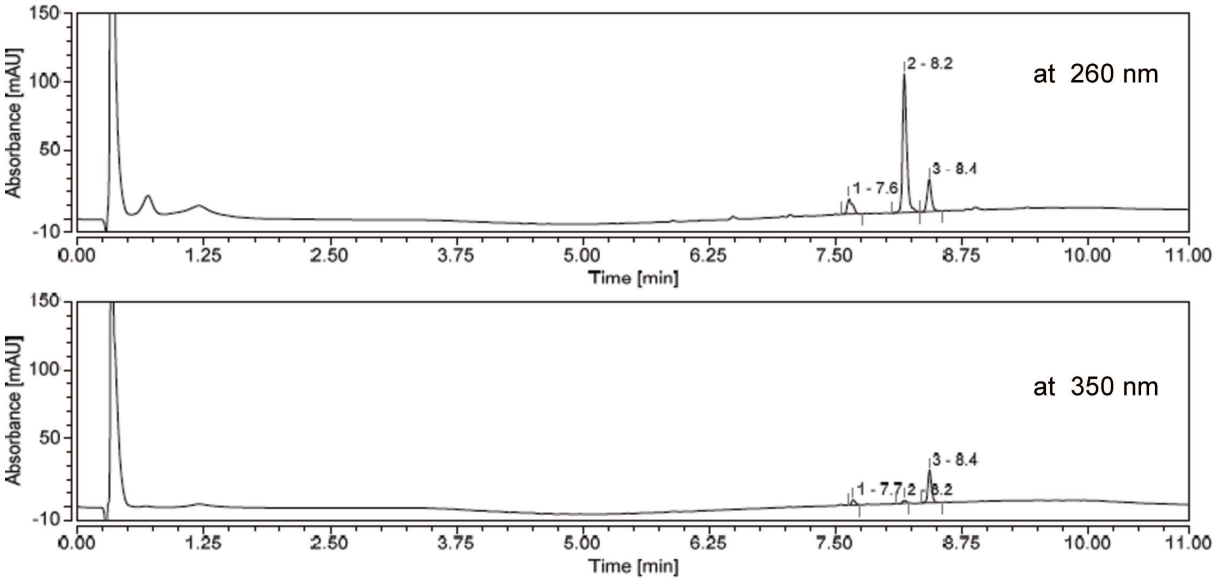
PLE-NTR probe **8** with PLE (0.55 U) after 120 min (addition of 0.6 U NTR at 80 min)



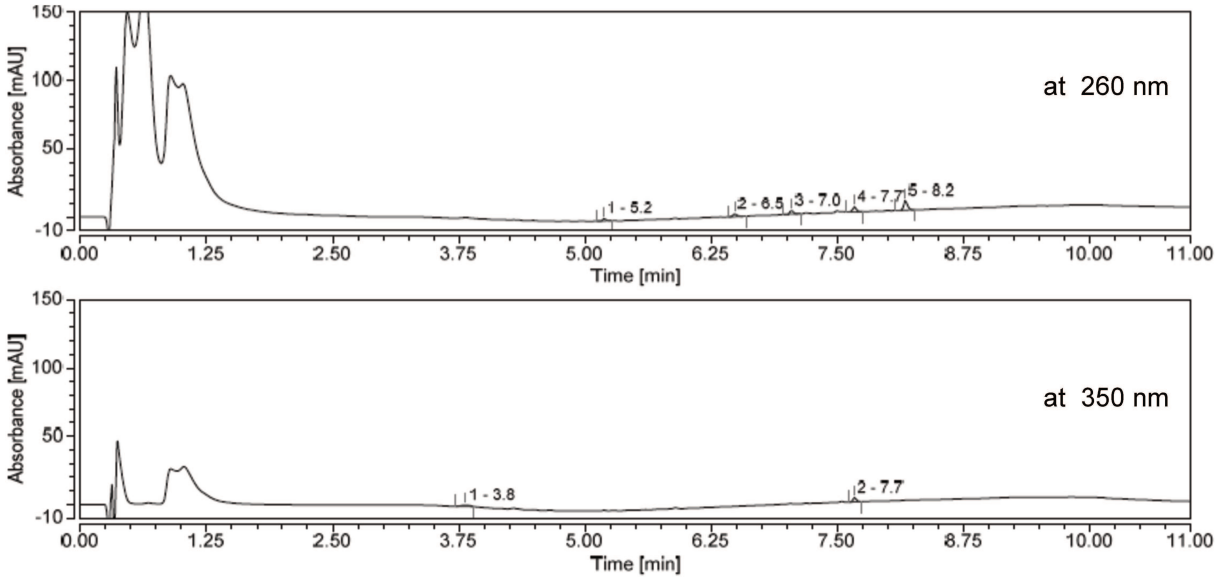
PLE-NTR probe **8** with PLE (0.55 U) after 180 min (addition of 0.6 U NTR at 80 min)



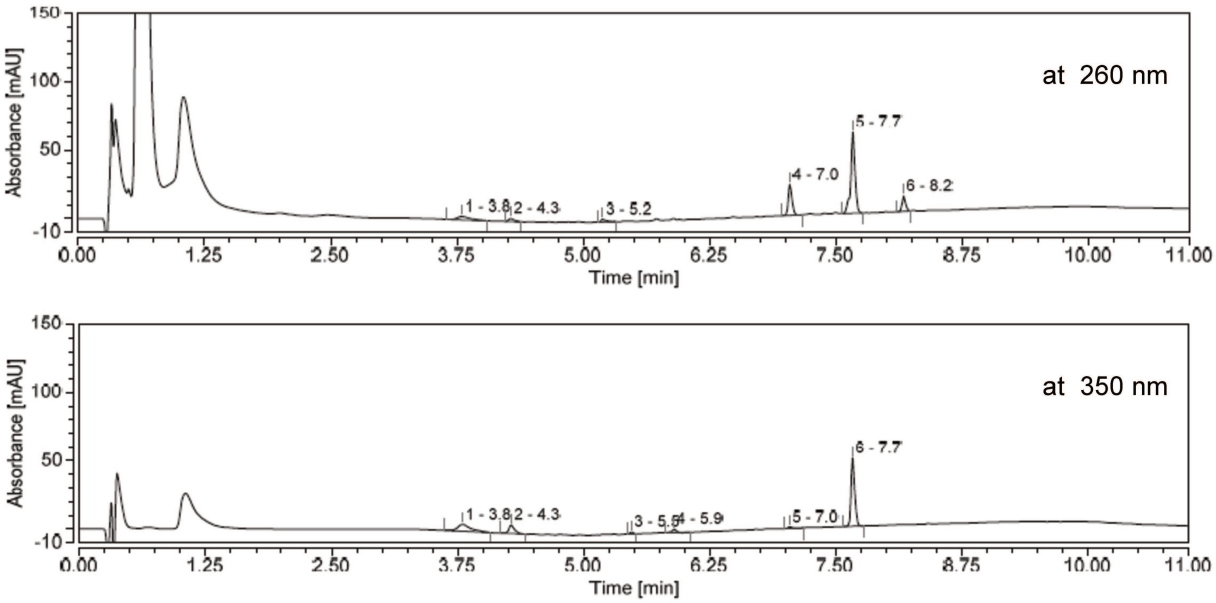
PLE-NTR probe **8** with NTR (0.6 U) after 30 min



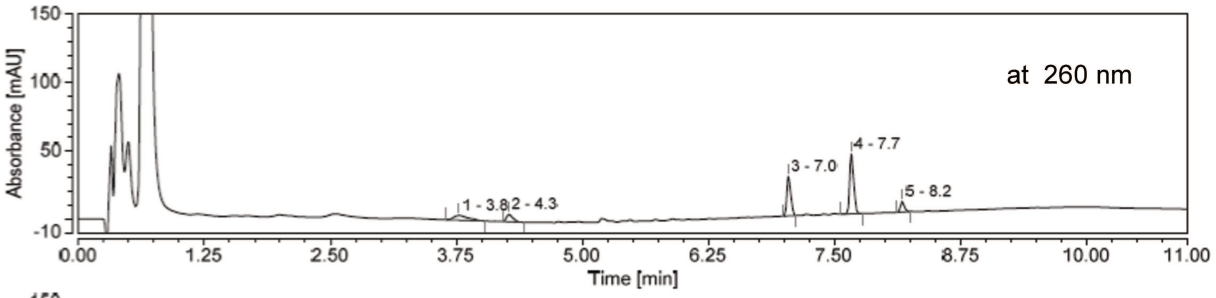
PLE-NTR probe **8** with NTR (0.6 U) after 60 min

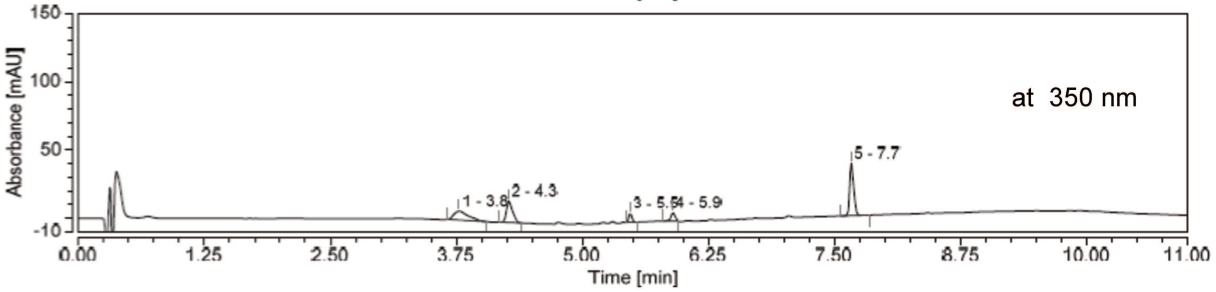


PLE-NTR probe **8** with NTR (0.6 U) after 120 min (addition of 0.55 U PLE at 80 min)

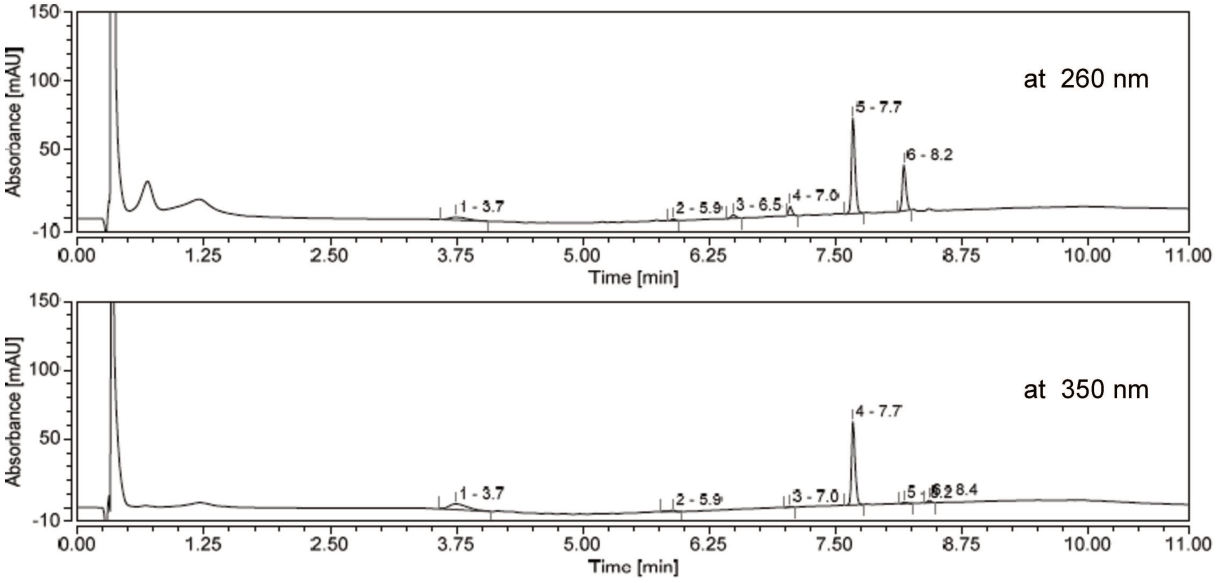


PLE-NTR probe **8** with NTR (0.6 U) after 180 min (addition of 0.55 U PLE at 80 min)

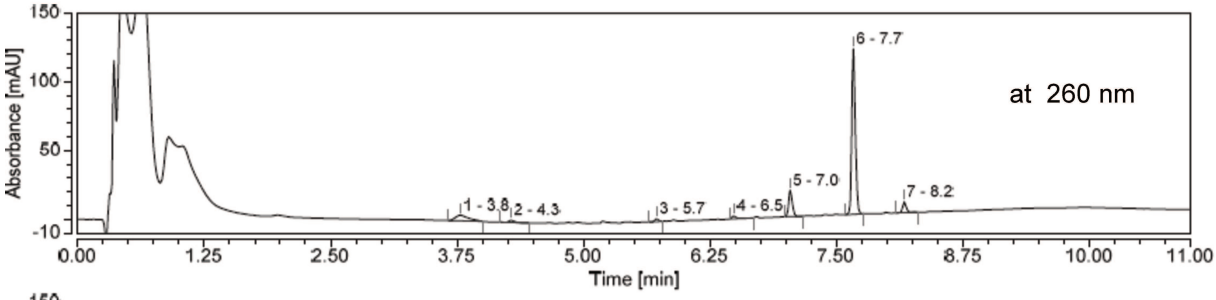


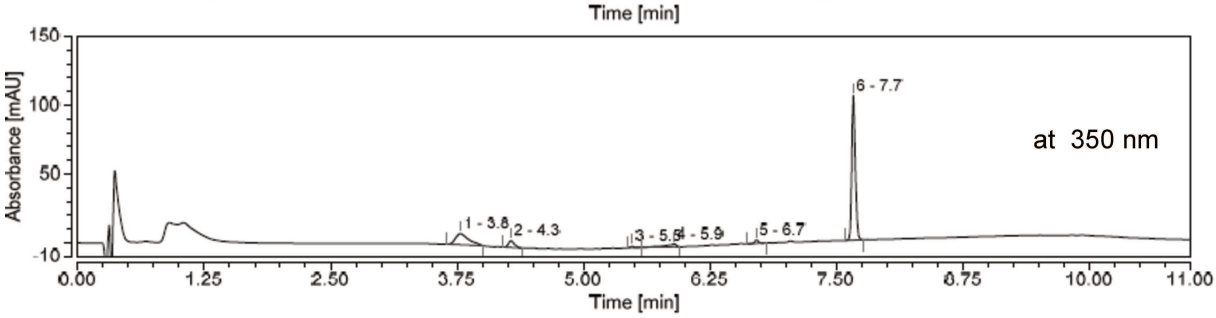


PLE-NTR probe **8** with NTR (0.6 U) and PLE (0.55 U) after 30 min

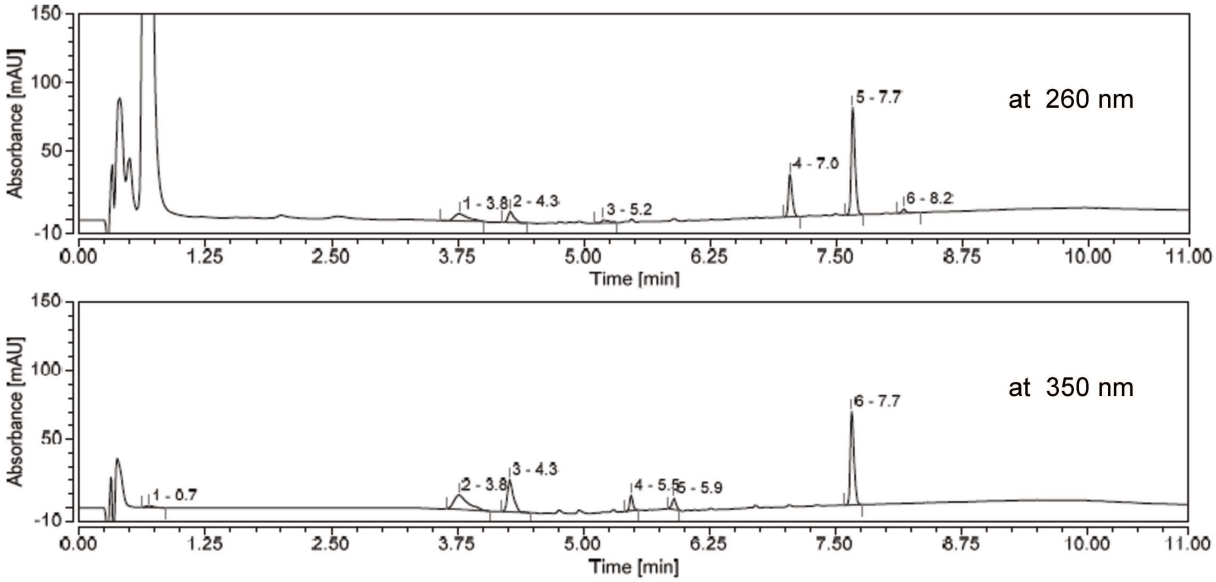


PLE-NTR probe **8** with NTR (0.6 U) and PLE (0.55 U) after 60 min





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

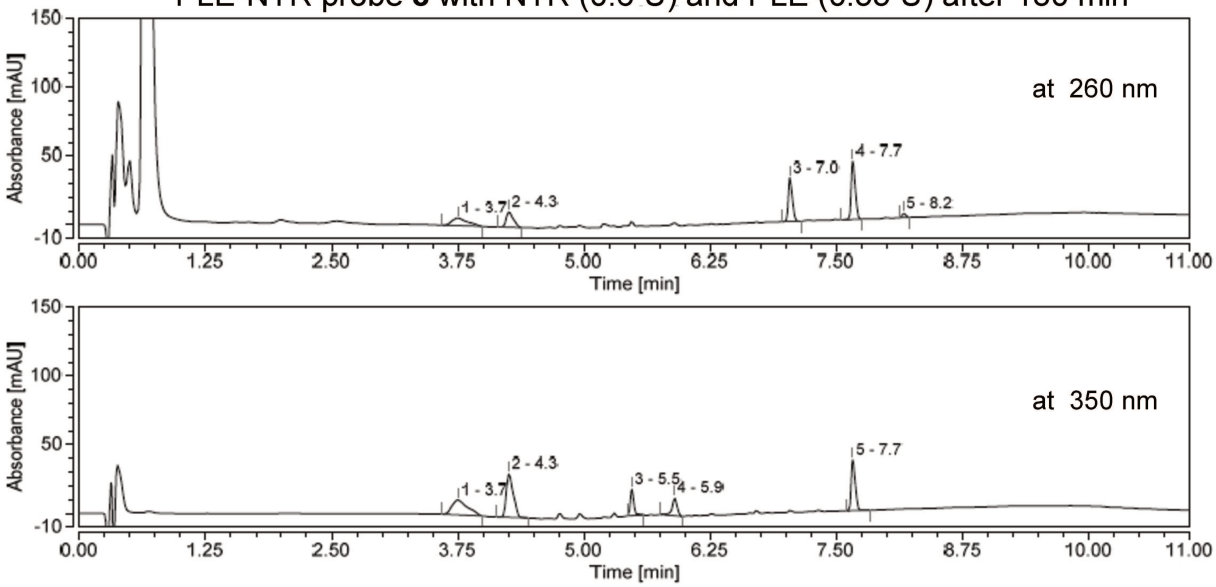
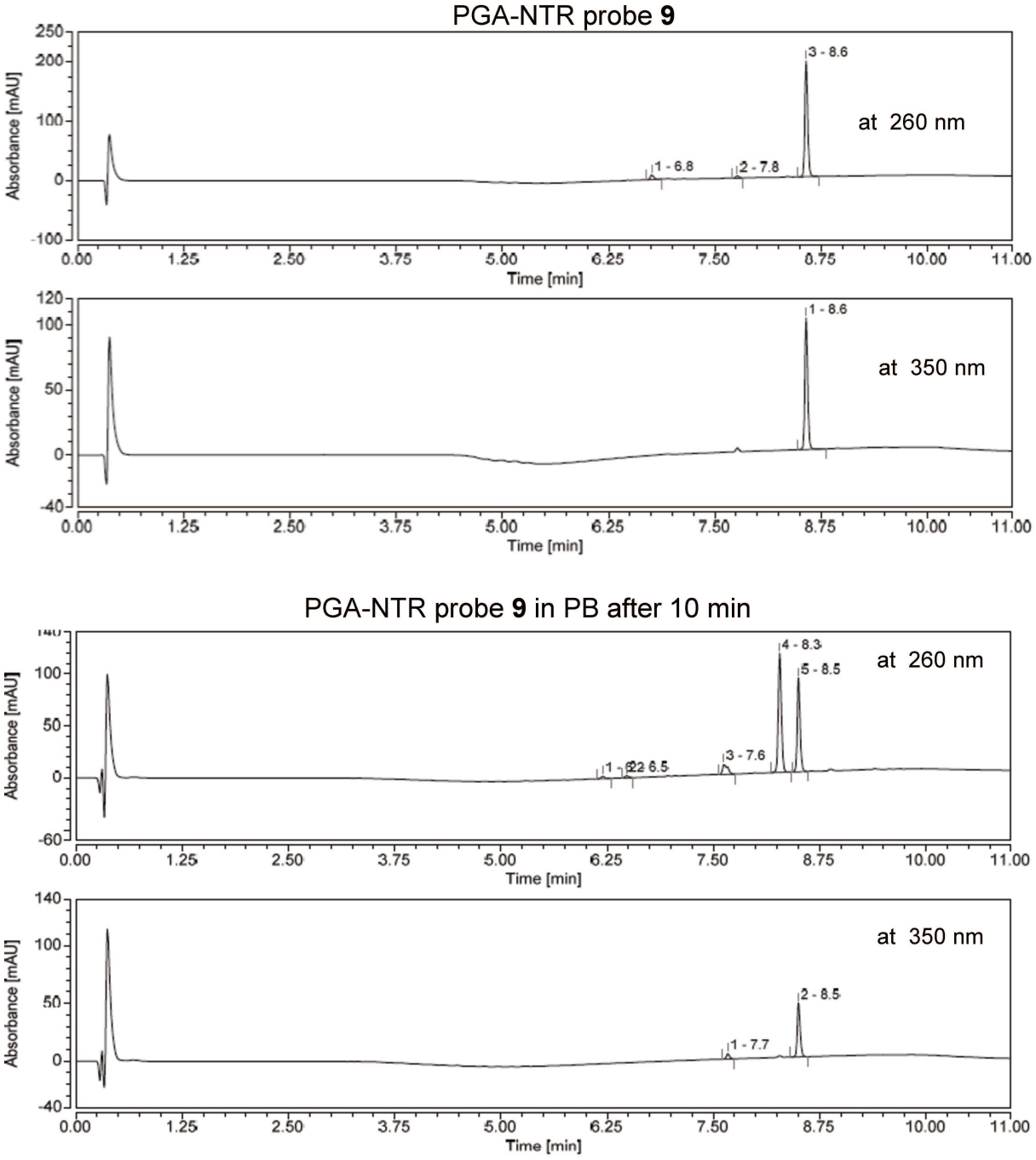
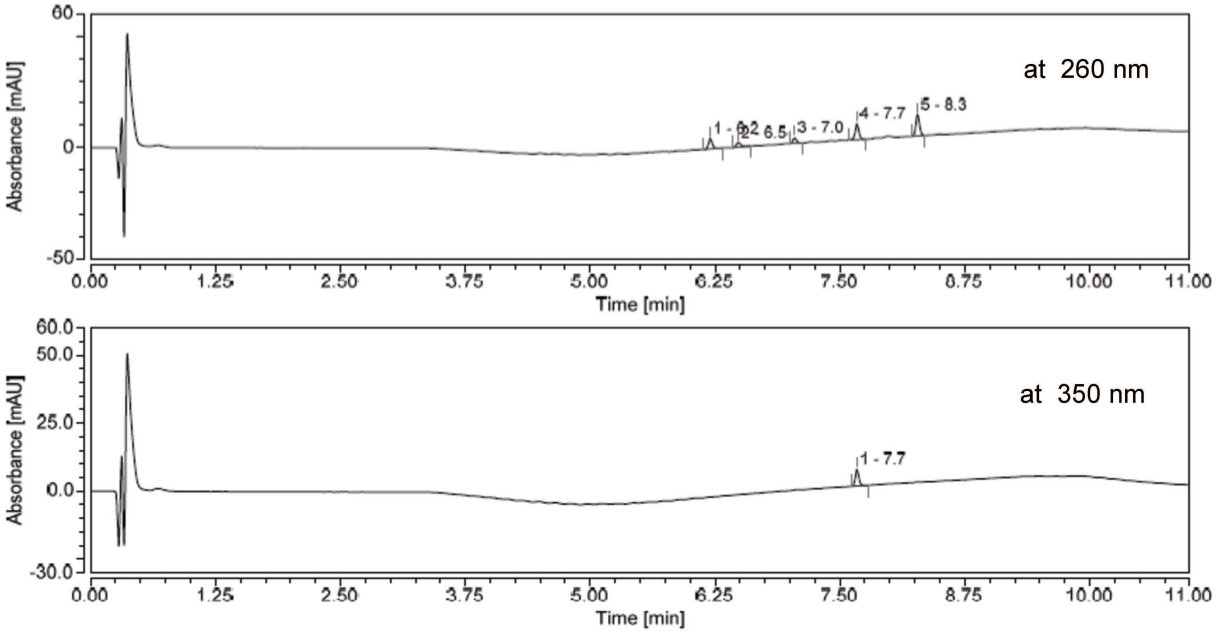


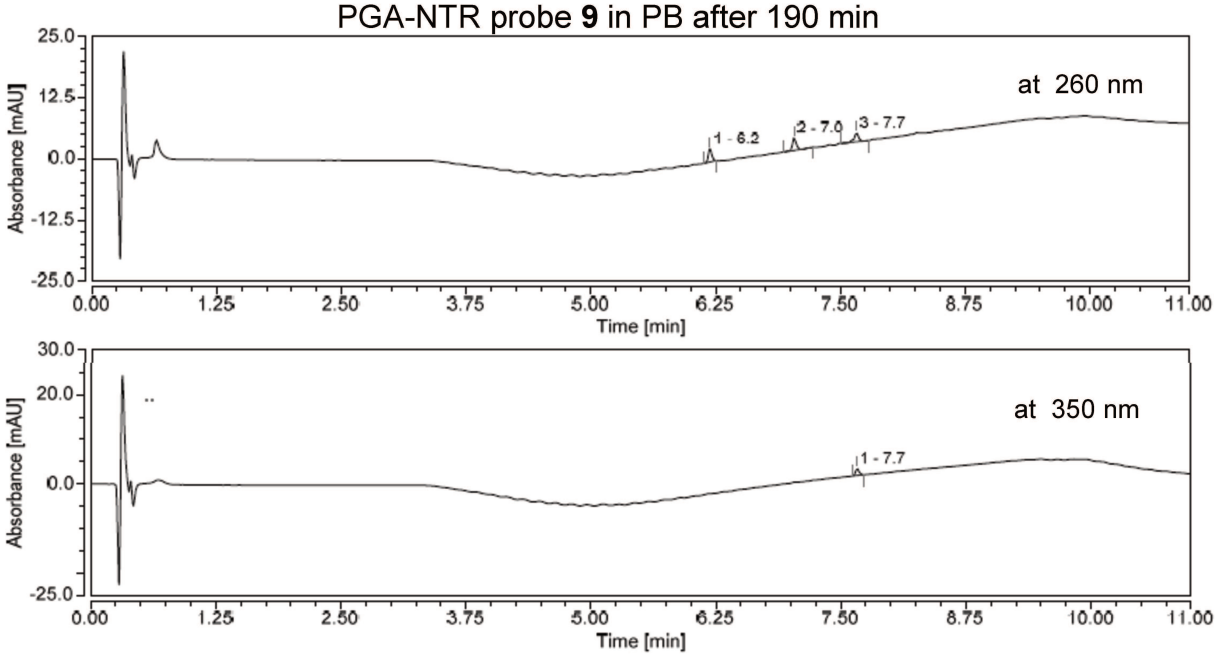
Fig S12. RP-HPLC elution profiles (system C) of fluorogenic probe 9 after incubation in PB alone or with enzymes (PGA and NTR/NADH)



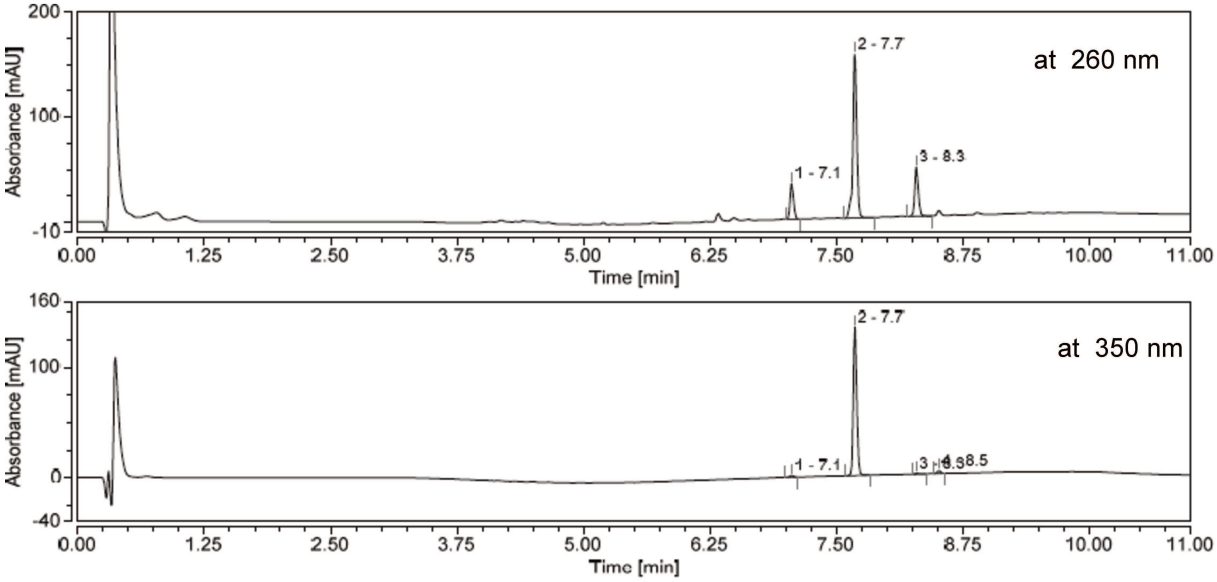
PGA-NTR probe **9** in PB after 90 min



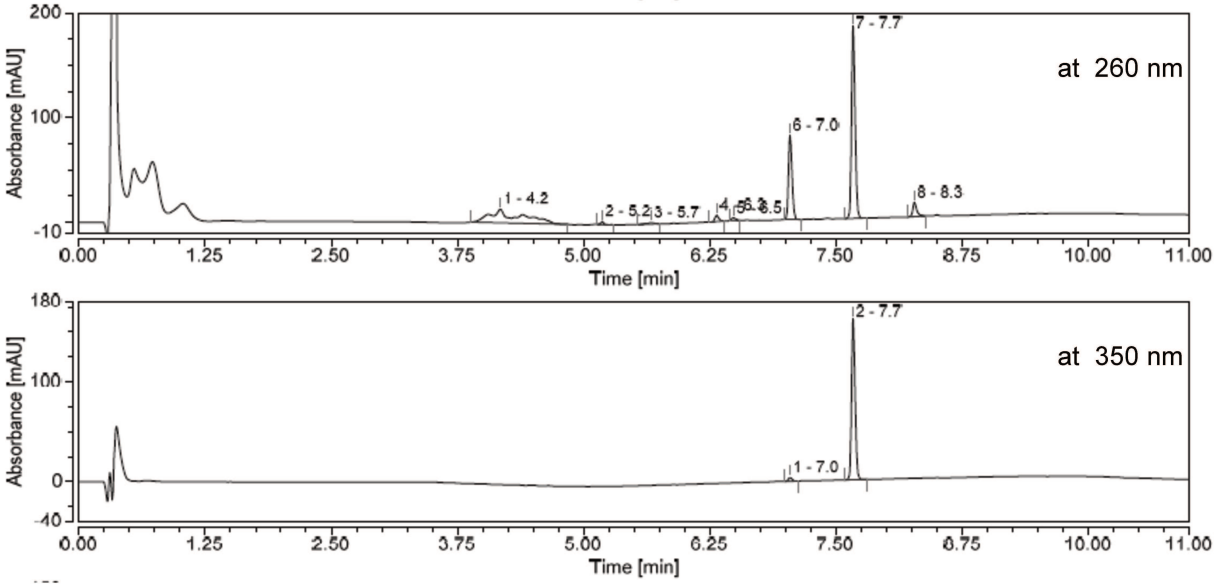
PGA-NTR probe **9** in PB after 190 min



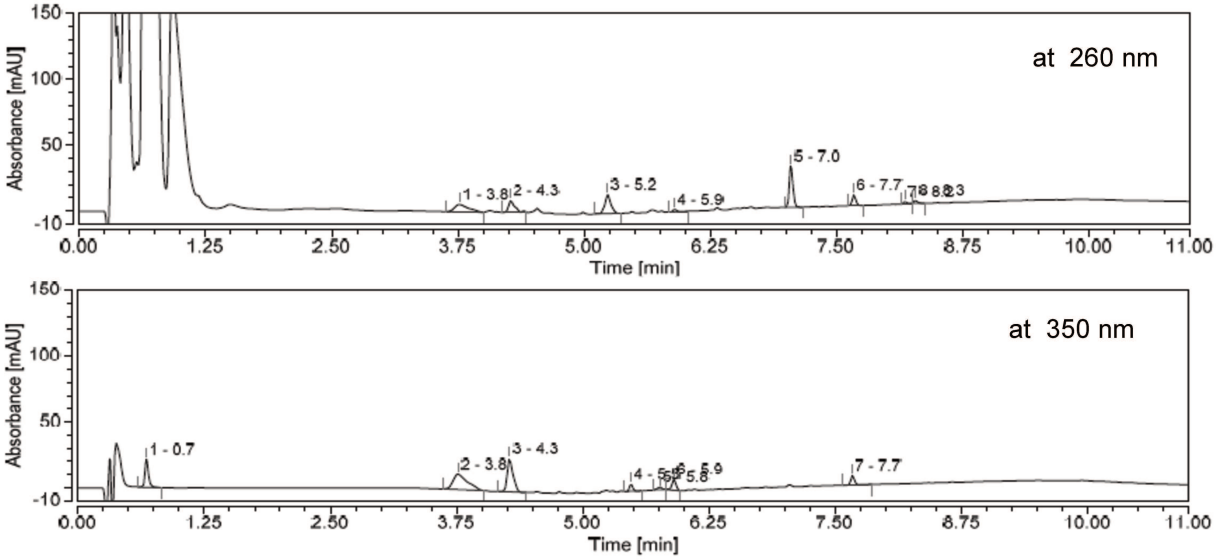
PGA-NTR probe **9** with PGA (0.75 U) after 30 min



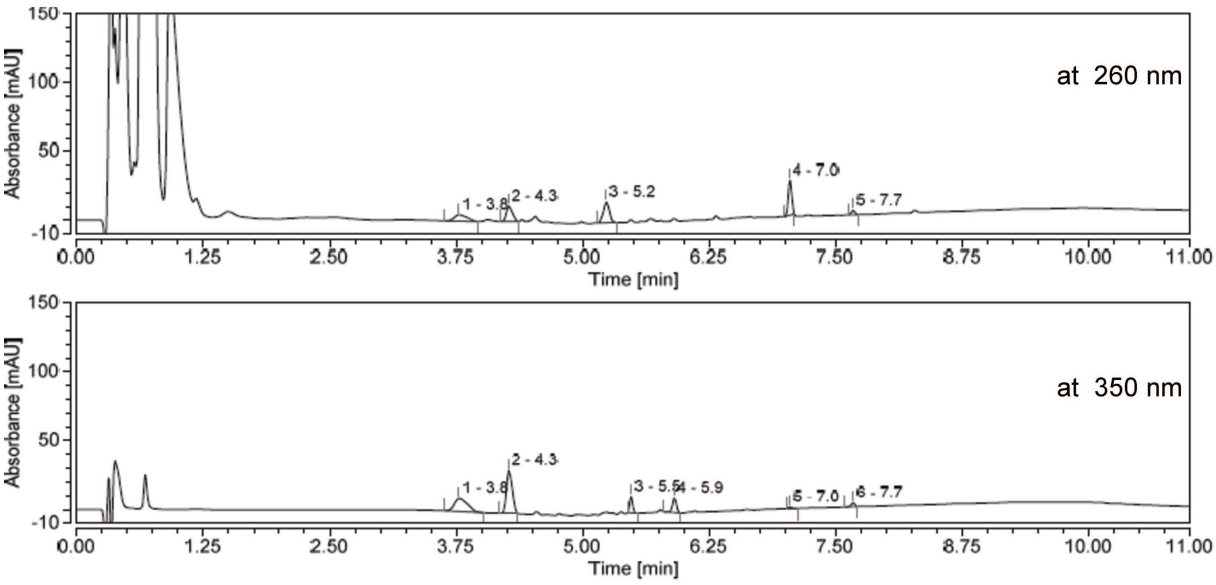
PGA-NTR probe **9** with PGA (0.75 U) after 60 min



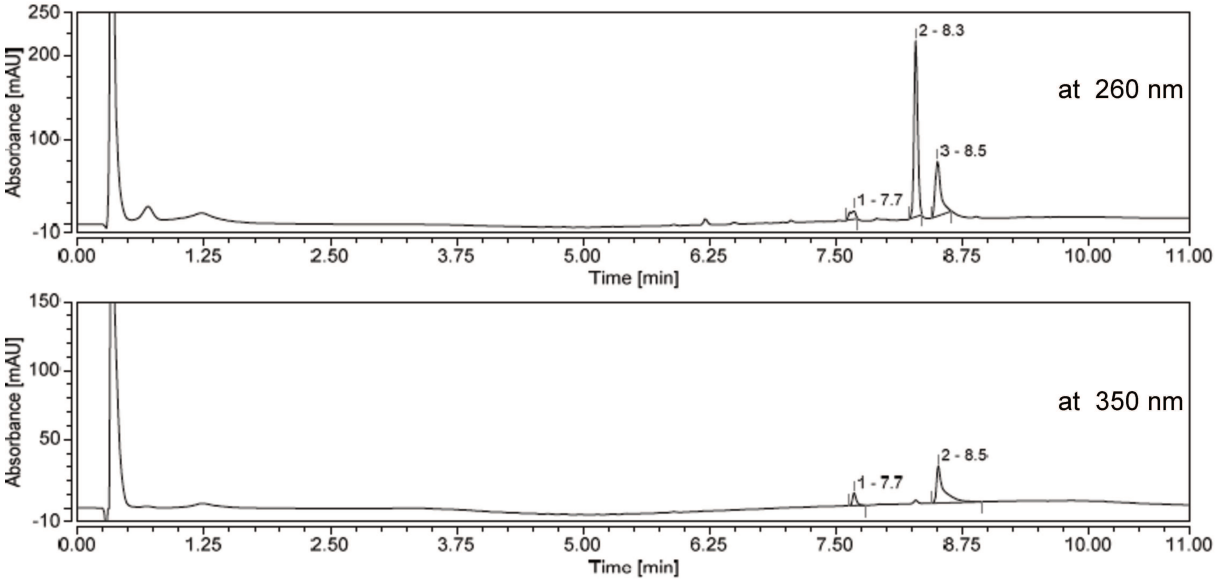
PGA-NTR probe **9** with PGA (0.75 U) after 120 min (0.6 U NTR adding at 80 min)



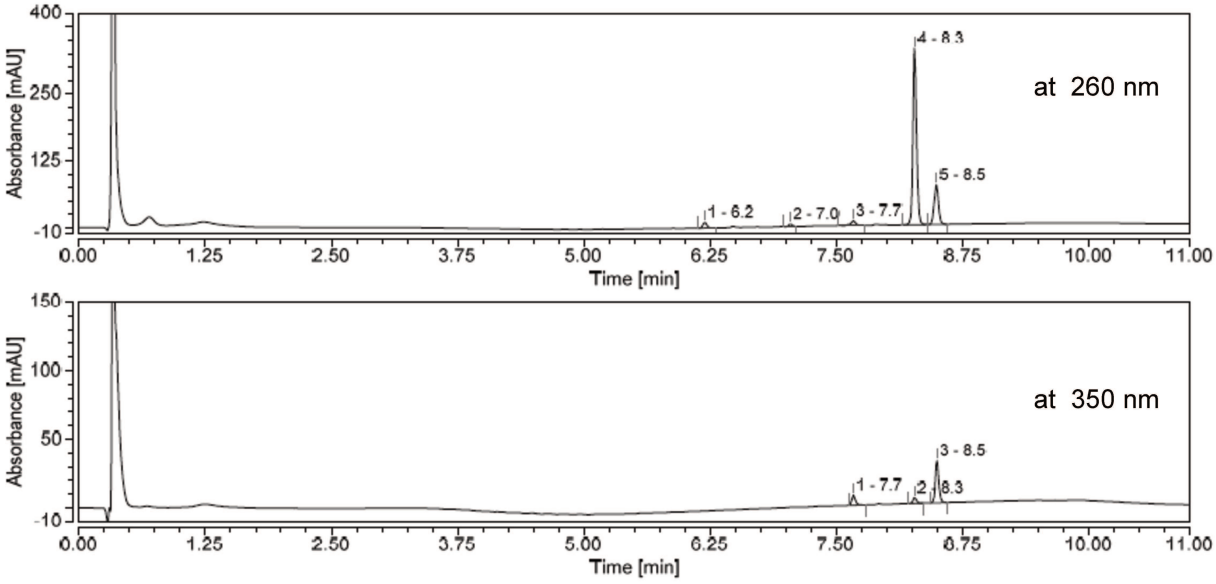
PGA-NTR probe **9** with PGA (0.75 U) after 180 min (0.6 U NTR addition at 80 min)



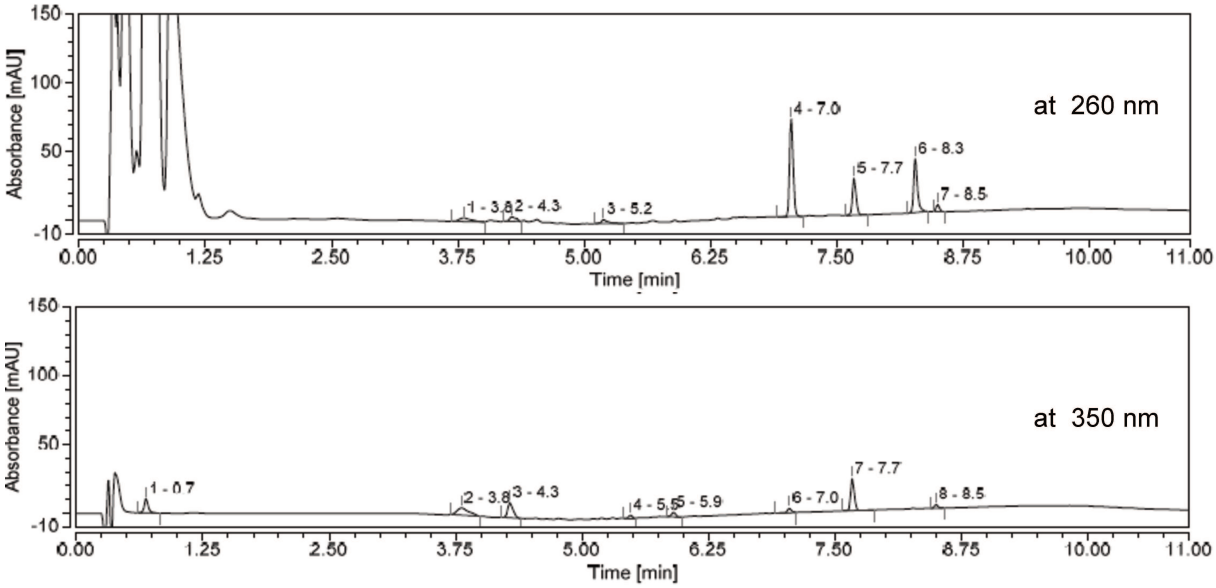
PGA-NTR probe **9** with NTR (0.6 U) after 30 min



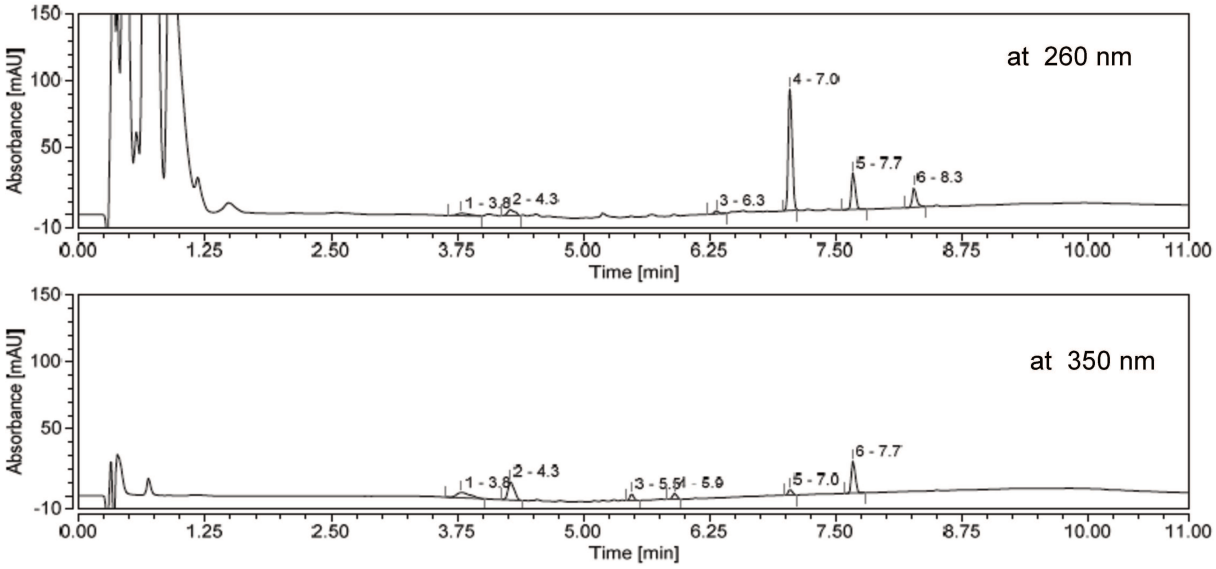
PGA-NTR probe **9** with NTR (0.6 U) after 60 min



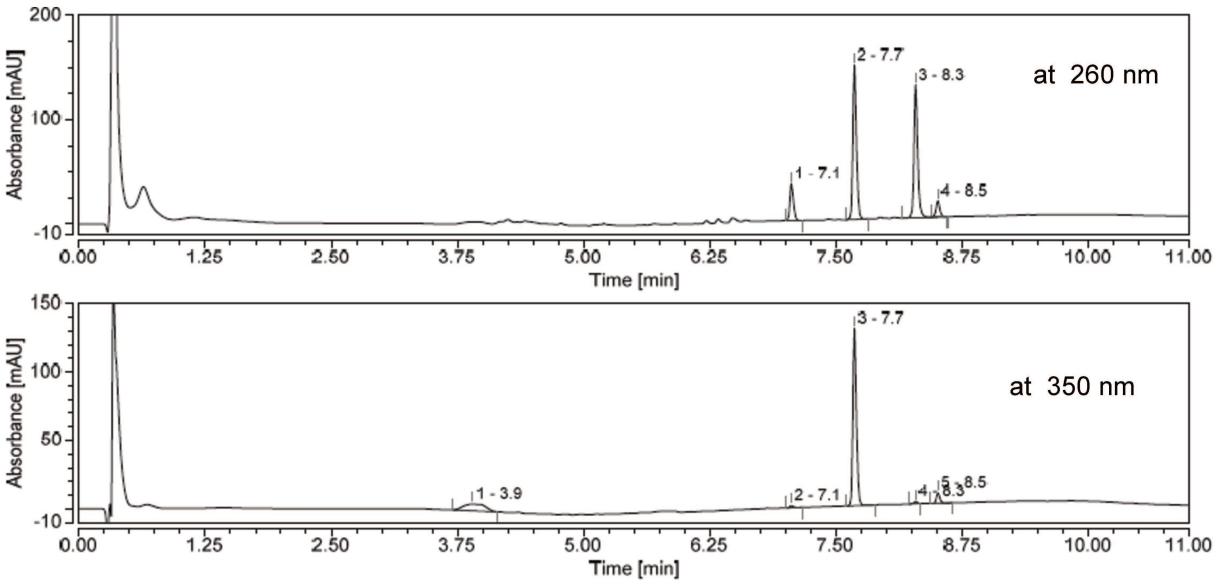
PGA-NTR probe **9** with NTR (0.6 U) after 120 min (addition of 0.75 U PGA at 80 min)



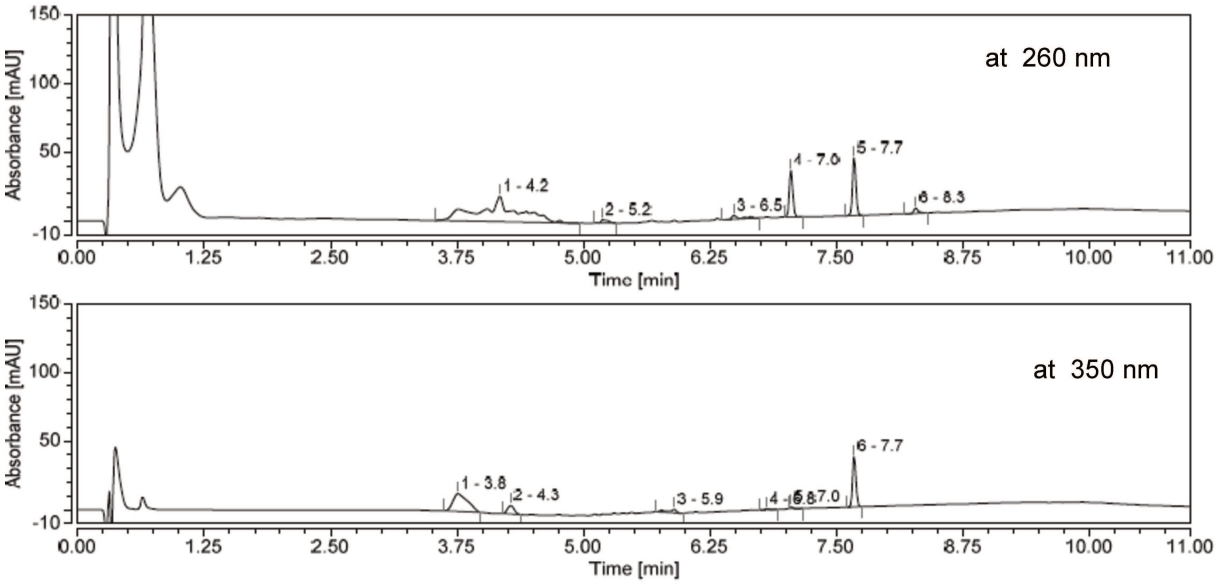
PGA-NTR probe **9** with NTR (0.6 U) after 180 min (addition of 0.75 U PGA at 80 min)



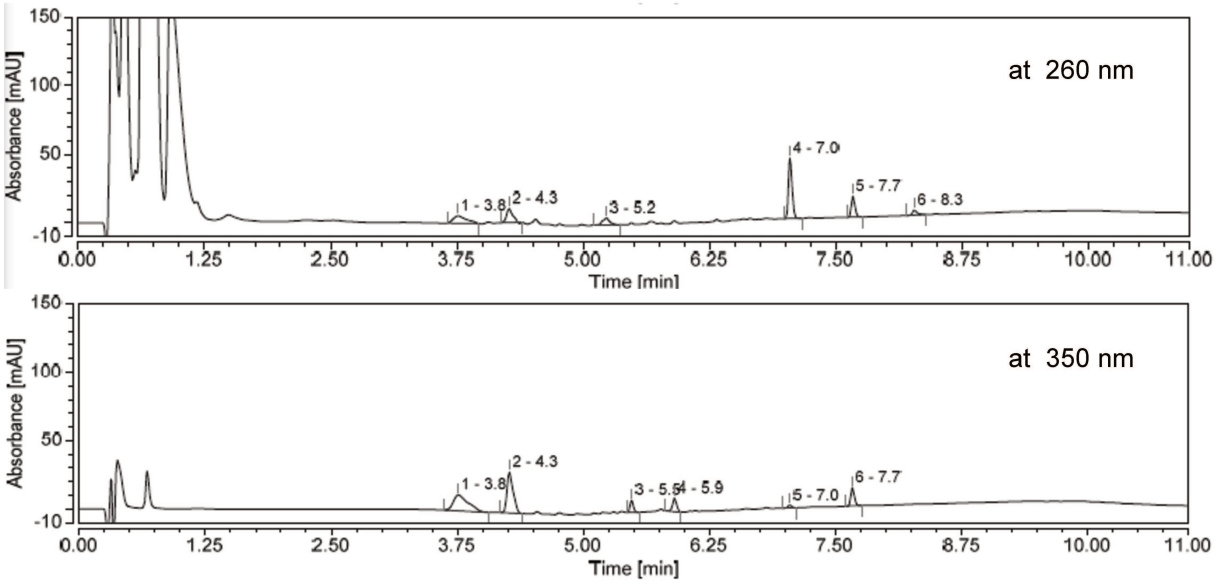
PGA-NTR probe **9** with NTR (0.6 U) and PGA (0.75 U) after 30 min



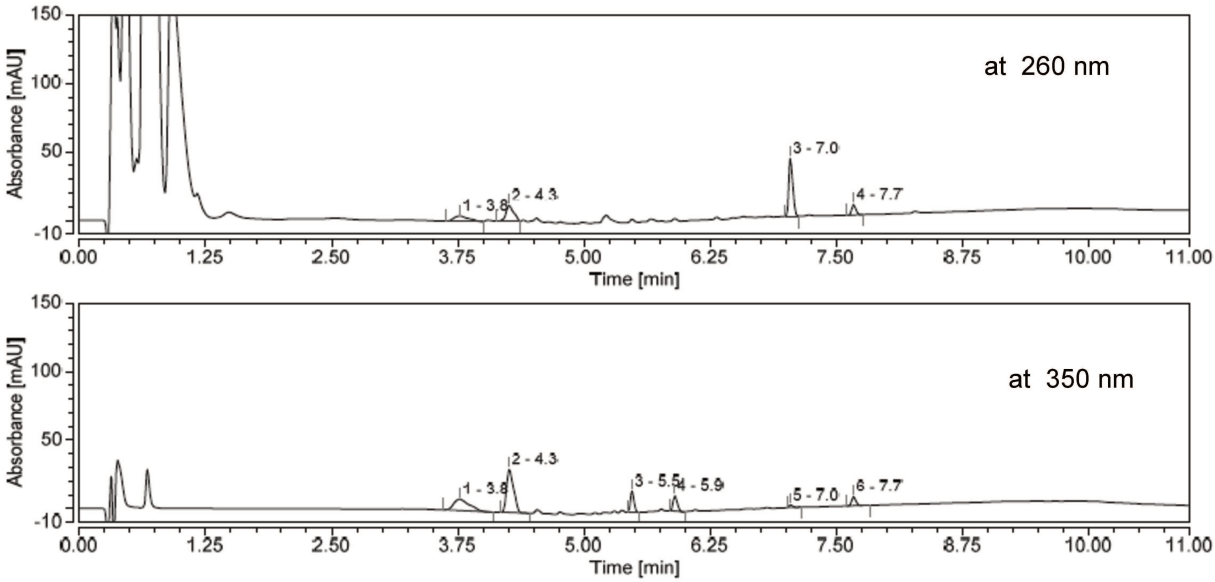
PGA-NTR probe **9** with NTR (0.6 U) and PGA (0.75) after 60 min



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



SI.2.2. Experimental section and "Supporting Information" file of Article 2: In situ formation of pyronin dyes for fluorescence protease sensing

Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

In situ formation of pyronin dyes for fluorescence protease sensing

Sylvain Debieu[a] and Anthony Romieu[*a,b]

[a] *Institut de Chimie Moléculaire de l'Université de Bourgogne, UMR 6302, CNRS, Univ. Bourgogne Franche-Comté, 9 Avenue Alain Savary, 21078 Dijon, France*
Fax: +33 (0)3 80 39 61 17
Tel: +33 (0)3 80 39 36 24
E-mail: anthony.romieu@u-bourgogne.fr
Lab homepage: http://www.icmub.fr

[b] *Institut Universitaire de France, 103 Boulevard Saint-Michel, 75005 Paris, France*

Supporting Information

Table of Contents

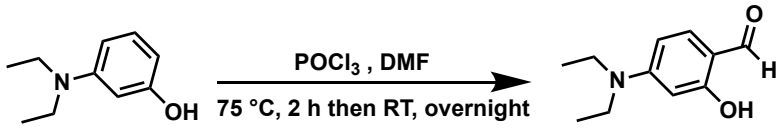
Abbreviations

The following abbreviations are used throughout the text of the ESI file: Abs, absorption; ATR, attenuated total reflectance; CH_3CN, acetonitrile; CH_3OH, methanol; DCM, dichloromethane; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; equiv., equivalent(s); Em, emission; EtOAc, ethyl acetate; EtOH, ethanol; ESI, electrospray ionisation; Ex, excitation; FA, formic acid; HPLC, high-pressure liquid chromatography; IR, infrared; LAP, leucyl-aminopeptidase; LRMS, low-resolution mass spectrum; min, minutes; $NaHCO_3$, sodium hydrogenocarbonate; NaOAc, sodium acetate; $(NH_4)_2SO_4$, ammonium sulfate; NMR, nuclear magnetic resonance; PB, phosphate buffer; PBS, phosphate buffered saline; PGA, penicillin G acylase; $MgCl_2$, magnesium chloride; MS, mass spectrometry; RP, reversed phase; rpm, revolution per minute; RT, room temperature; t_R, retention time; UV, ultraviolet; vis, visible.

Synthesised compounds

4-(Diethylamino)salicylaldehyde (1)[1]

(CAS number :17754-90-4)



Phosphorous oxychloride ($POCl_3$) (7.0 mL, 72.6 mmol, 3 equiv.) was added dropwise to dry DMF (9.5 ml, 121 mmol, 5 equiv.) at 0 °C. Then 3-(*N*,*N*-diethylamino)phenol (4.0 g, 24.2 mmol, 1 equiv.) in dry DMF (15 mL) was slowly added to the previous mixture and the resulting solution was heated at 75 °C for 2 h 30 and at RT overnight. Thereafter, the reaction mixture was cooled to RT, poured into ice cold water and neutralized with aq. saturated $NaHCO_3$ and then the solid was collected by filtration. The solid was retaken with DCM and filtrated over a plug of silica gel, eluted with DCM/EtOAc (9 : 1, v/v). The filtrate was evaporated under vaccum and the product **1** was cristalised in EtOH/ water (9 : 1, v/v) to afford **1** as white solid (3.9 g, yield 83%). IR (ATR): ν = 3374 (broad), 2974, 1624, 1558, 1517, 1490, 1446, 1414, 1378, 1335, 1303, 1236, 1217, 1186, 1125, 1096, 1077, 1012, 958, 869, 841, 802, 772, 721 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ = 1.12 (t, *J* = 7.2 Hz, 6 H, C$\underline{H}_3$-Et), 3.39 (q, *J* = 7.0 Hz, 4 H, C$\underline{H}_2$-Et), 6.04 (d, *J* = 2.4 Hz, 1 H, $\underline{H}$-Ar), 6.34 (dd, *J* = 2.4 Hz, *J* = 9.0 Hz, 1 H, $\underline{H}$-Ar), 7.42 (d, *J* = 9.0 Hz, 1 H, $\underline{H}$-Ar), 9.61 (s, 1 H, $\underline{H}$-formyl), 11.26 (s, 1 H, O$\underline{H}$) ppm; ^{13}C NMR (126 MHz, DMSO-d_6): δ = 12.4 (2 C, $\underline{C}H_3$-Et), 44.1 (2 C, $\underline{C}H_2$-Et), 95.9, 104.4, 111.2, 134.0, 153.8, 163.4, 190.6 ppm; HPLC (system A): t_R = 4.6 min (purity 100% at 260 nm and 360 nm); LRMS (ESI+, recorded

[1] V. S. Padalkar, A. Tathe, V. D. Gupta, V. S. Patil, K. Phatangare and N. Sekar, *J. Fluoresc.*, 2012, **22**, 311-322.

during RP-HPLC analysis): *m/z* 194.2 $[M + H]^+$ (100) and 235.2 $[M + H + CH_3CN]^+$ (30), calcd for $C_{11}H_{16}NO_2^+$ 194.1; LRMS (ESI-, recorded during RP-HPLC analysis): *m/z* 192.1 $[M - H]^-$ (20), calcd for $C_{11}H_{14}NO_2^-$ 192.1.

In vitro activation of fluorogenic "turn-on" probes 4 and 5 by PGA and LAP - experimental details

Stock solutions of probes and enzymes:

- Mixture A: A stock solution (1.0 mg / mL) of PGA fluorogenic probe **4** in HPLC-grade CH_3CN (final concentration: 2.48 mM),
- Mixture B: A stock solution (1.0 mg / mL) of LAP fluorogenic probe **5** in H_2O/CH_3CN (1 : 1, v/v) (final concentration: 1.47 mM),
- Mixture C: Commercial **PGA** (841 U / mL) directly used without dilution,
- Mixture D: 14.2 µL of the commercial **LAP** suspension was dissolved in 985.8 µL of ultrapure water (1 U / mL),
- Mixture E: 5.43 mg of ammonium sulfate in 825 µL of water (6.58 mg / mL, 50 mM)
- Mixture F: 25 µL of a $MgCl_2$ stock solution (prepared with 0.48 mg of $MgCl_2$ in 505 µL of water (0.95 mg / mL)) diluted in 475 µL of water (0.05 mM),

Stock solutions (1.0 mg / mL) of **6** were also prepared in DMSO (for spectroscopy, 99.9%, ACROS, 167852500) and subsquently diluted with PBS for UV-vis absorption and fluorescence measurements, and PB for HPLC-fluorescence analyses.

Fluorescence assays:

All assay were performed at 37 °C (using a Peltier temperature controler and water circulation, and conducted with magnetic stirring, rpm = 300). For both probes **4** and **5**, the fluorescence emission of the release 6-*N*,*N*-diethylamino-3*H*-xanthen-3-imine was monitored at λ = 545 nm (bandwidth = 5 nm) (Ex. λ = 525 nm, bandwidth = 5 nm) over time with measurements recorded every 5 s.

Protocol:

Probe **4** - Into a 3.5 mL fluorescence quartz cell, 1.2 µL of mixture A was diluted in 3 mL of selected aq. buffer (final concentration of probe: 1 µM) with additive (none or 2 µL of E or 6 µL of F or both) and the resulting mixture was incubated for 5 min. Then 1.2 µL of enzyme solution (mixture C, 1 U) was added.

Probe **5** - Into a 3.5 mL fluorescence quartz cell, 2 µL of mixture B was diluted in 3 mL of selected aq. buffer (final concentration of probe: 1 µM) and the resulting mixture was incubated for 5 min. Then, 2 µL of enzyme solution (mixture D, 0.002 U) was added.

Protocol for negative controls:

Probe **4** - Into a 3.5 mL fluorescence quartz cell, 1.2 µL of mixture A was diluted in 3 mL of PB (100 mM, pH 7.6) and the resulting mixture was incubated for 5 min. Then 2 µL of **LAP** solution (mixture D, 0.002 U) was added.

Probe **5** - Into a 3.5 mL fluorescence quartz cell, 2 µL of mixture B was diluted in 3 mL of selected PB (100 mM, pH 7.6) and the resulting mixture was incubated for 5 min. Then, 1.2 µL of **PGA** solution (mixture C, 1 U) was added.

HPLC-fluorescence analyses:

Enzymatic reaction mixtures from fluorescence assays were directly analysed by RP-HPLC-fluorescence after an incubation time of 24 h (injected volume: 10 µL, system H).

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

Incubation protocols:

Probe **4** - 39 nmol (16 µL of mixture A) fluorogenic PGA-sensitive probe was dissolved in selected aq. buffer (470 µL) containing 1.2 µL of PGA solution (mixture C, 1 U) and the resulting enzymatic reaction mixture was incubated at 37 °C for 24 h.

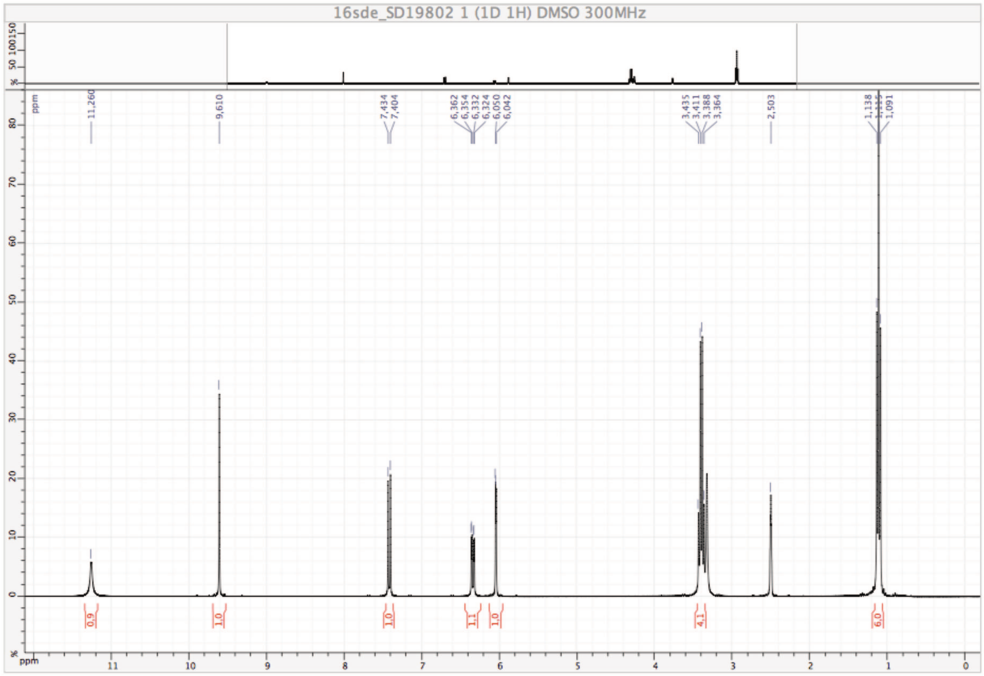
Probe **5** - 39 nmol (27 µL of mixture B) fluorogenic LAP-sensitive probe was dissolved in selected aq. buffer (420 µL) containing 50 µL of LAP solution (mixture D, 0.05 U) and the resulting enzymatic reaction mixture was incubated at 37 °C for 24 h. Samples (50 µL) were taken at 30 min, 2 h, 5 h, 8 h and 24 h of reaction and were treated as described below.

Samples treatment for HPLC-MS analysis:

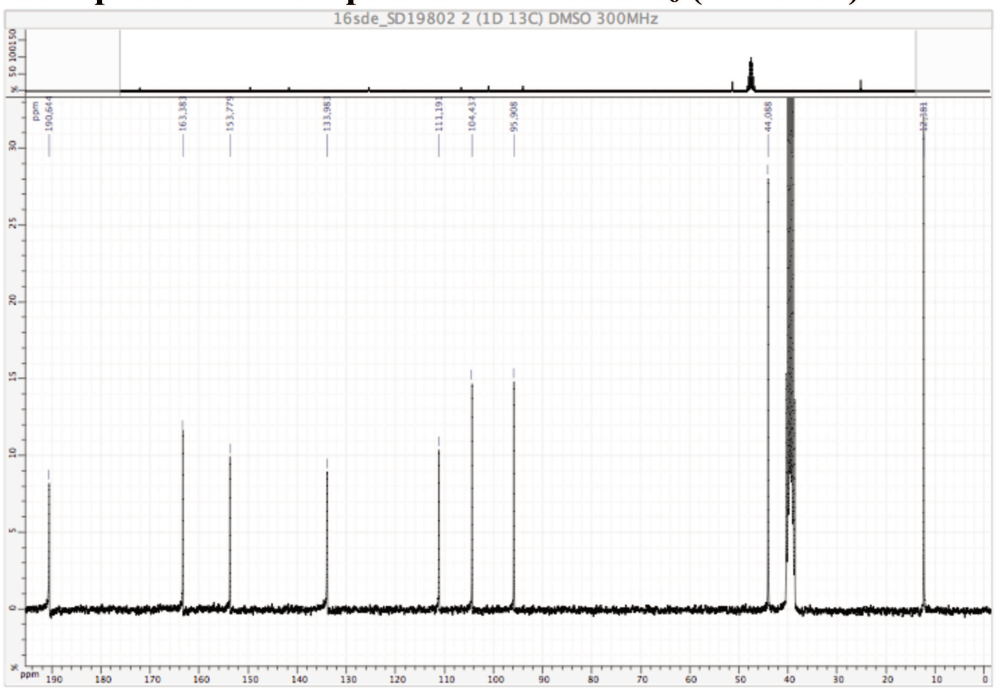
Withdrawn sample (50 µL) was diluted with 50 µL of CH_3CN, then vortexed followed by centrifugation (9 000 rpm, 2 min) and finally, 75 µL of the supernatant was collected and diluted with 25 µL of ultrapure H_2O. 15 µL was injected into the HPLC-MS apparatus (system G).

Analytical data

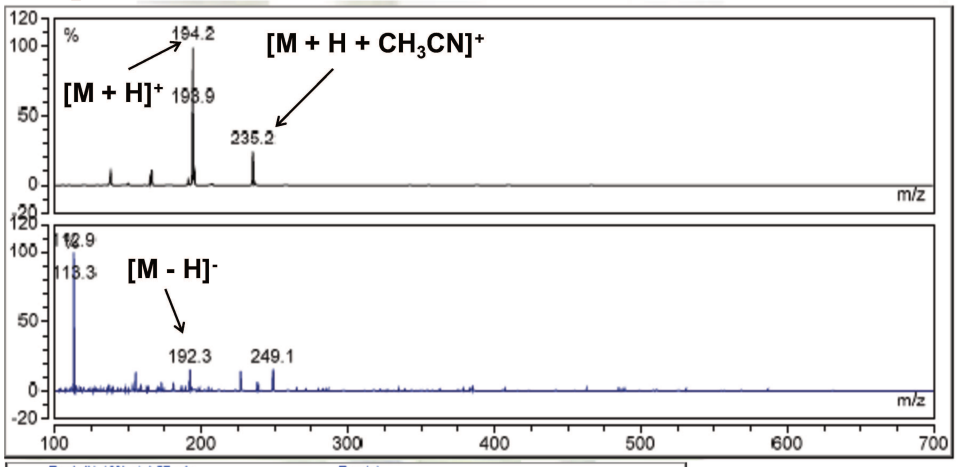
^{1}H NMR spectrum of compound 1 in DMSO-d_6 (300 MHz)

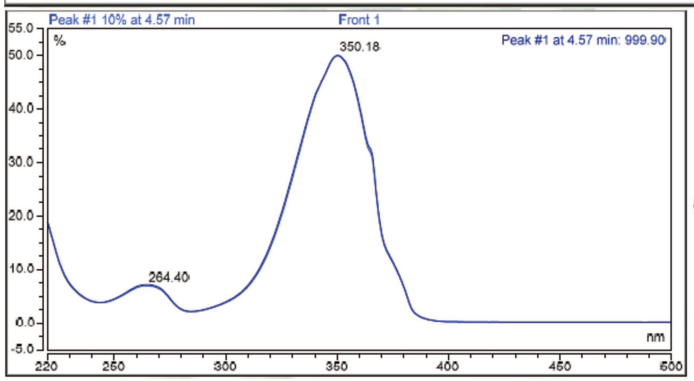


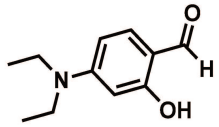
^{13}C NMR spectrum of compound 1 in DMSO-d_6 (126 MHz)



ESI+ / ESI- mass spectra (low resolution) and UV-vis spectrum of compound 1

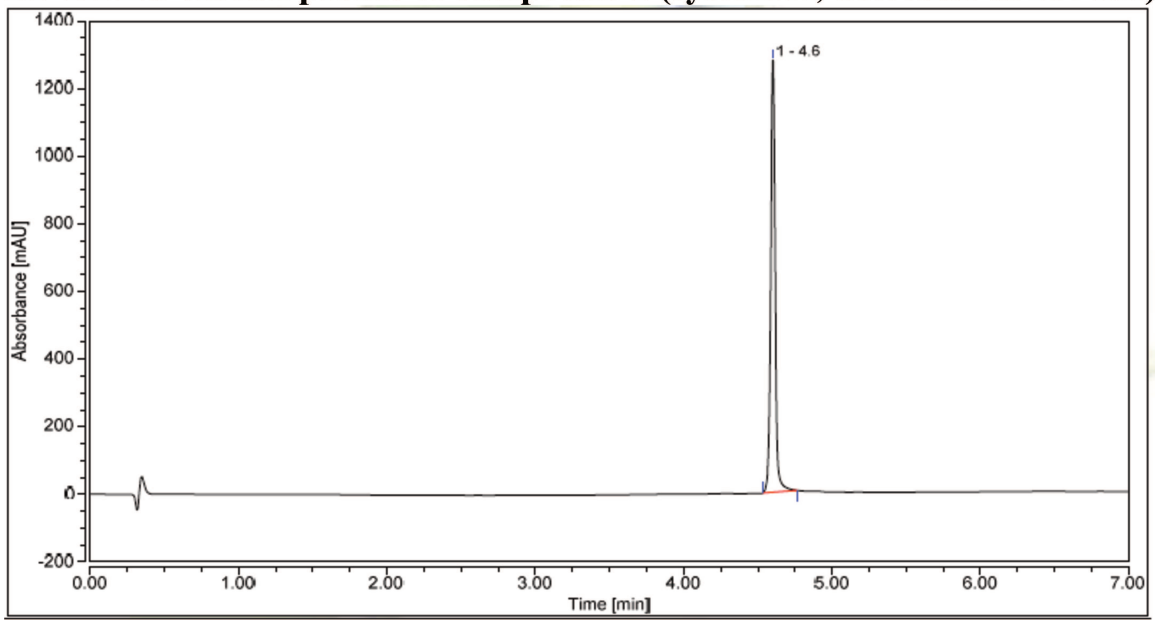




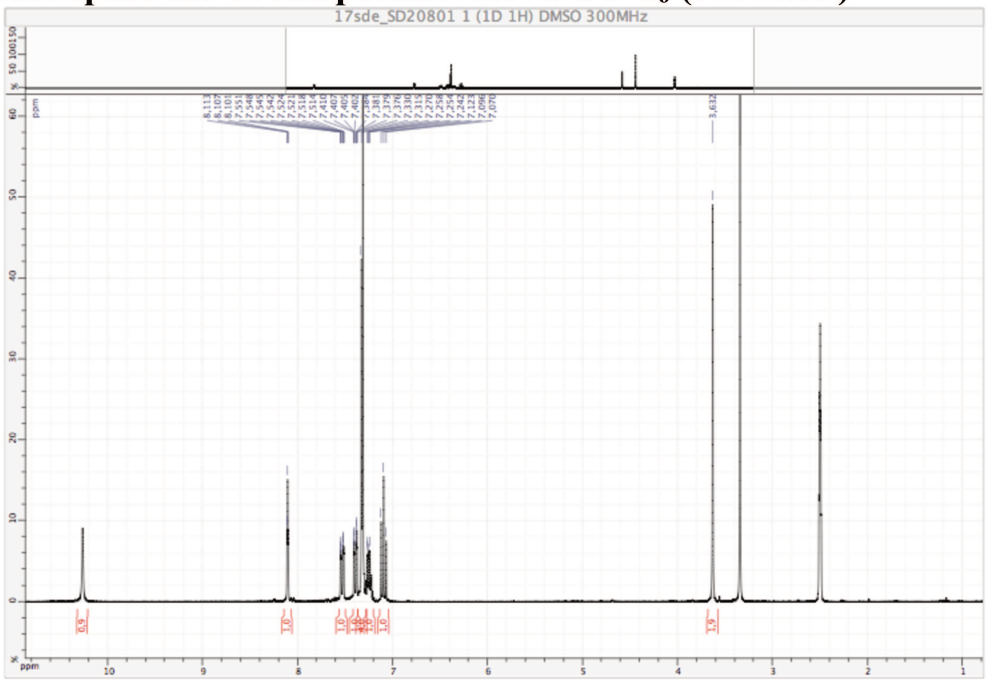


Chemical Formula: $C_{11}H_{15}NO_2$
Molecular Weight: 193,25

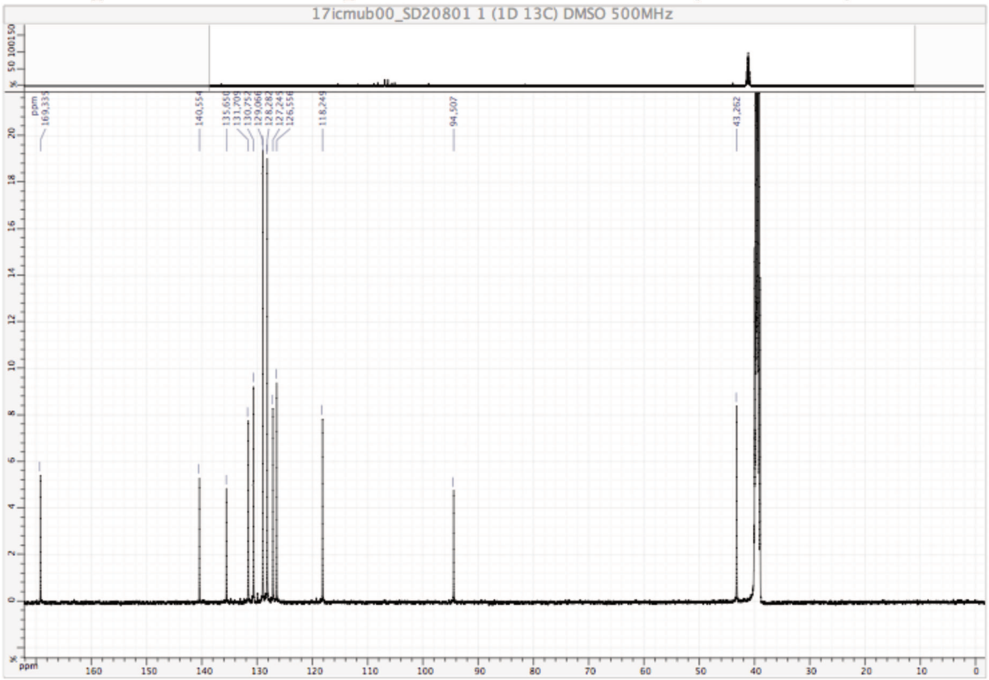
RP-HPLC elution profile of compound 1 (system A, detection at 260 nm)



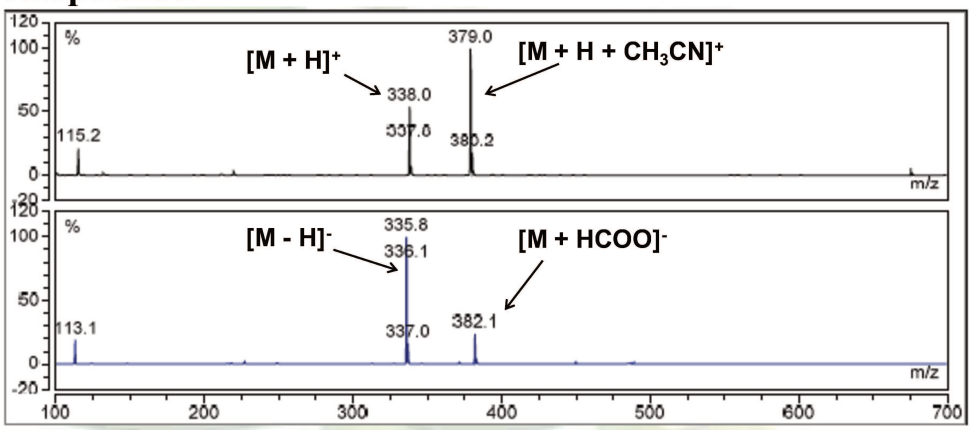
^{1}H NMR spectrum of compound 2 in DMSO-d_6 (300 MHz)

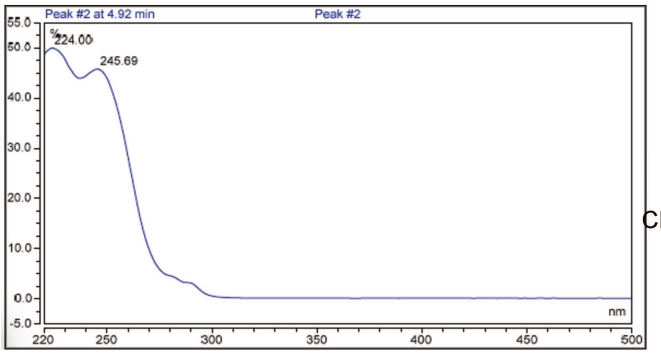


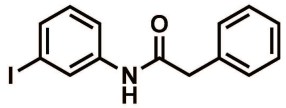
^{13}C NMR spectrum of compound 2 in DMSO-d_6 (126 MHz)



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

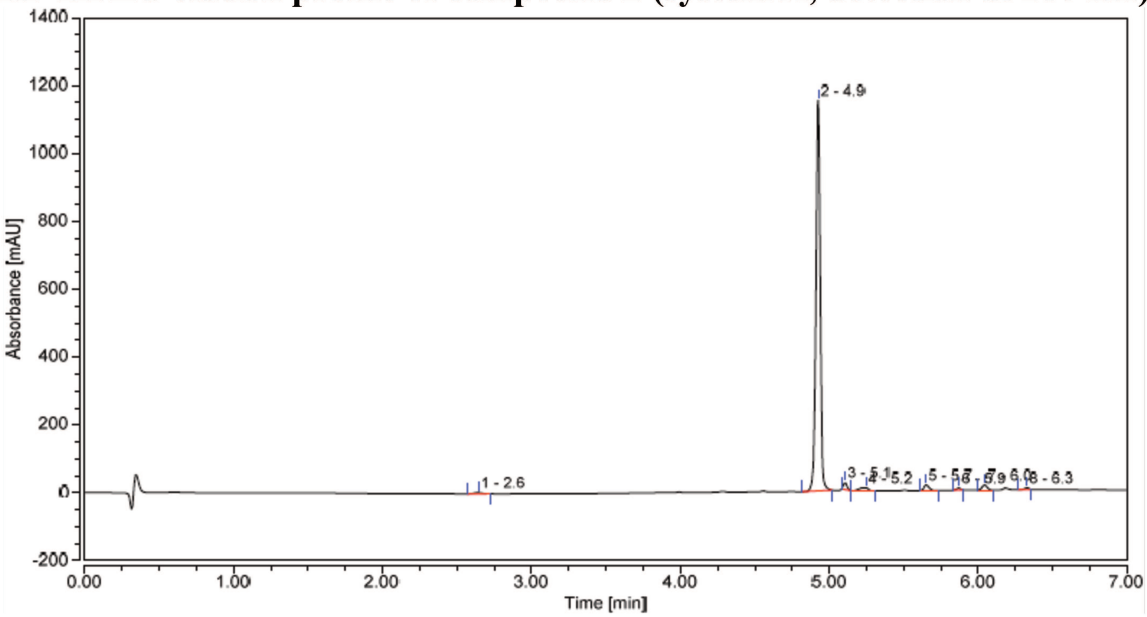




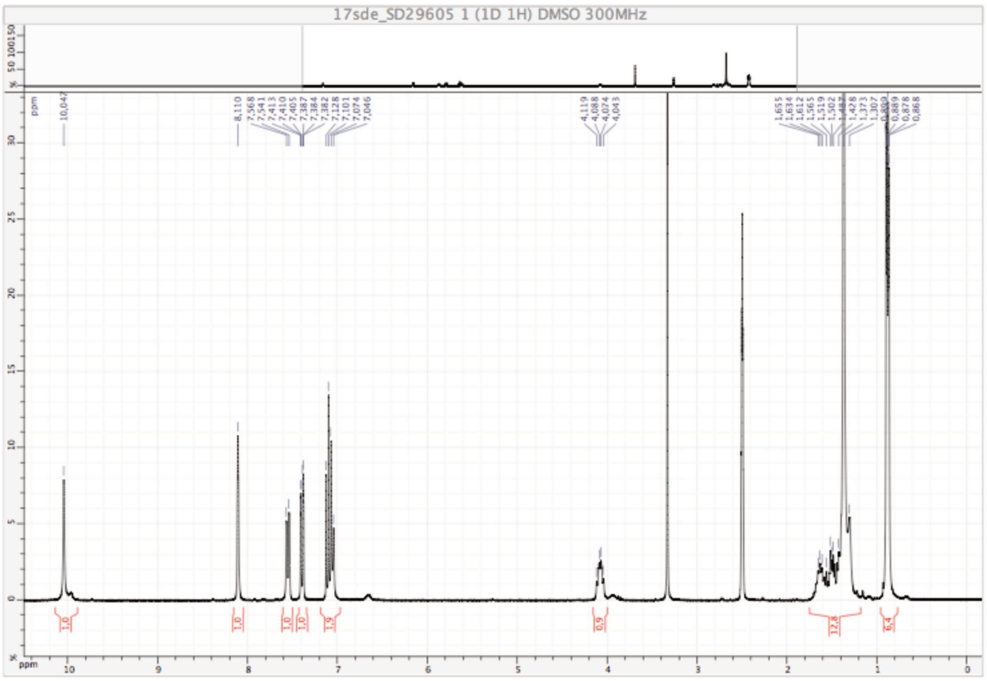


Chemical Formula: $C_{14}H_{12}INO$
Molecular Weight: 337,16

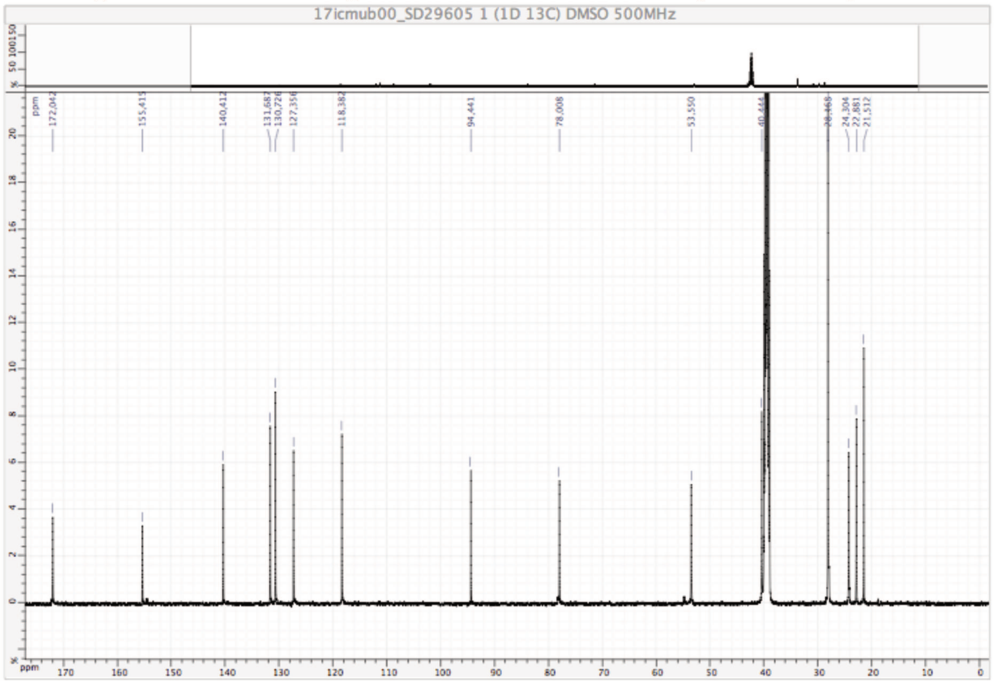
RP-HPLC elution profile of compound 2 (system A, detection at 260 nm)



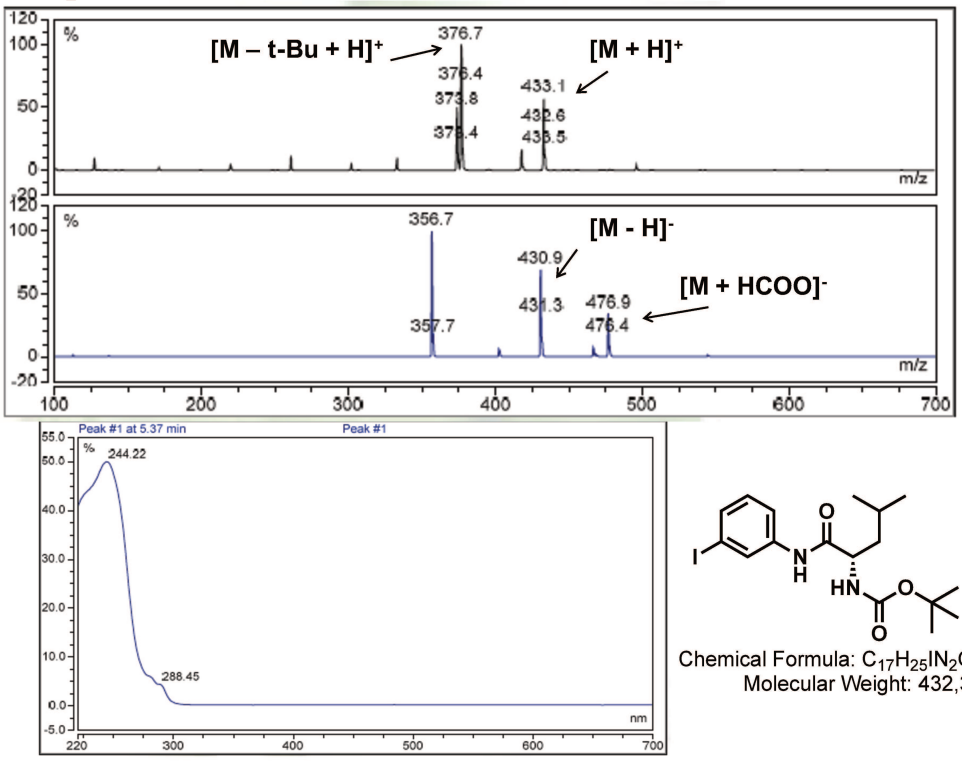
^{1}H NMR spectrum of compound 3 in DMSO-d_6 (300 MHz)



^{13}C NMR spectrum of compound 3 in DMSO-d_6 (126 MHz)

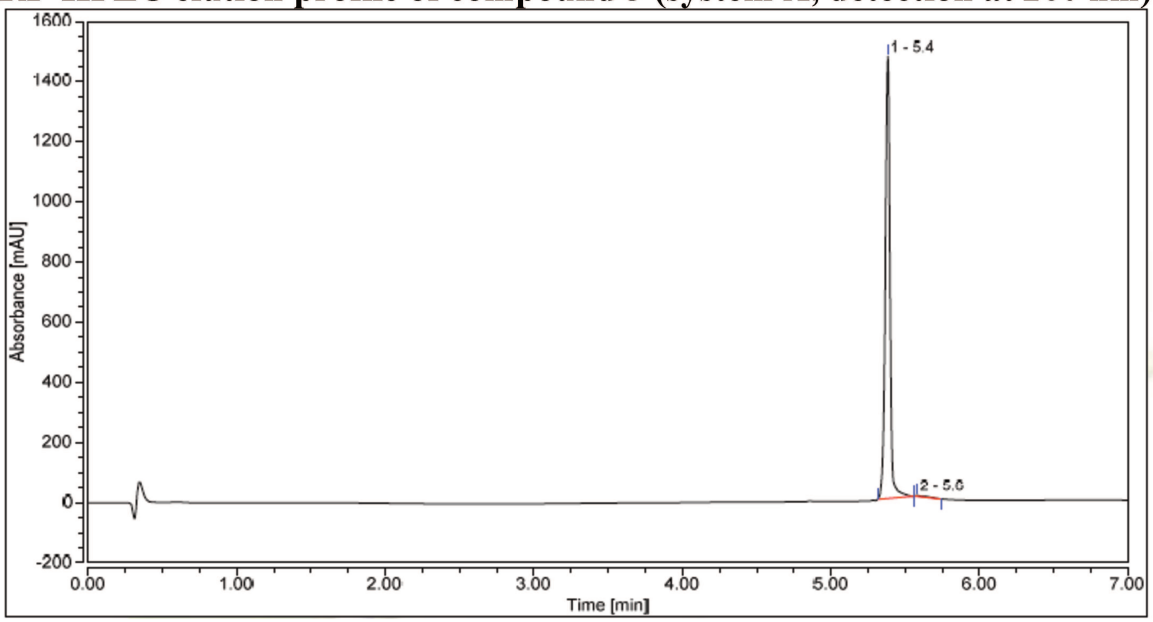


ESI+ / ESI- mass spectra (low resolution) and UV-vis spectrum of compound 3

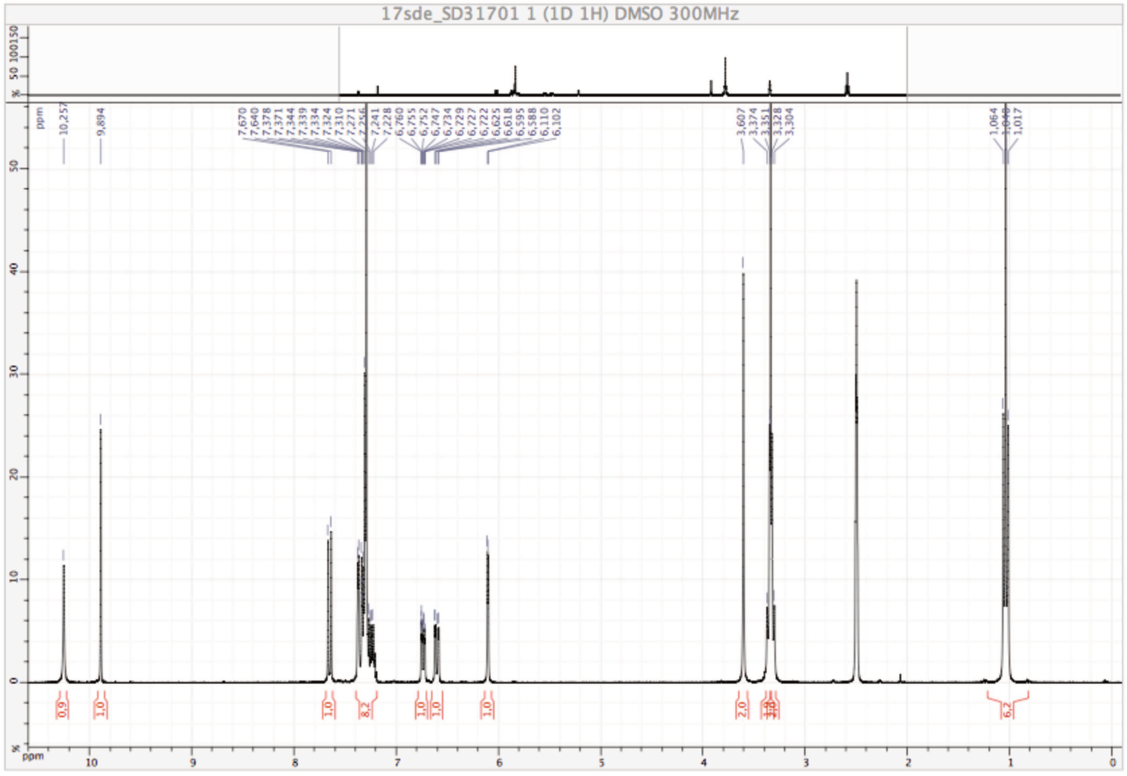


Chemical Formula: $C_{17}H_{25}IN_2O_3$
Molecular Weight: 432,30

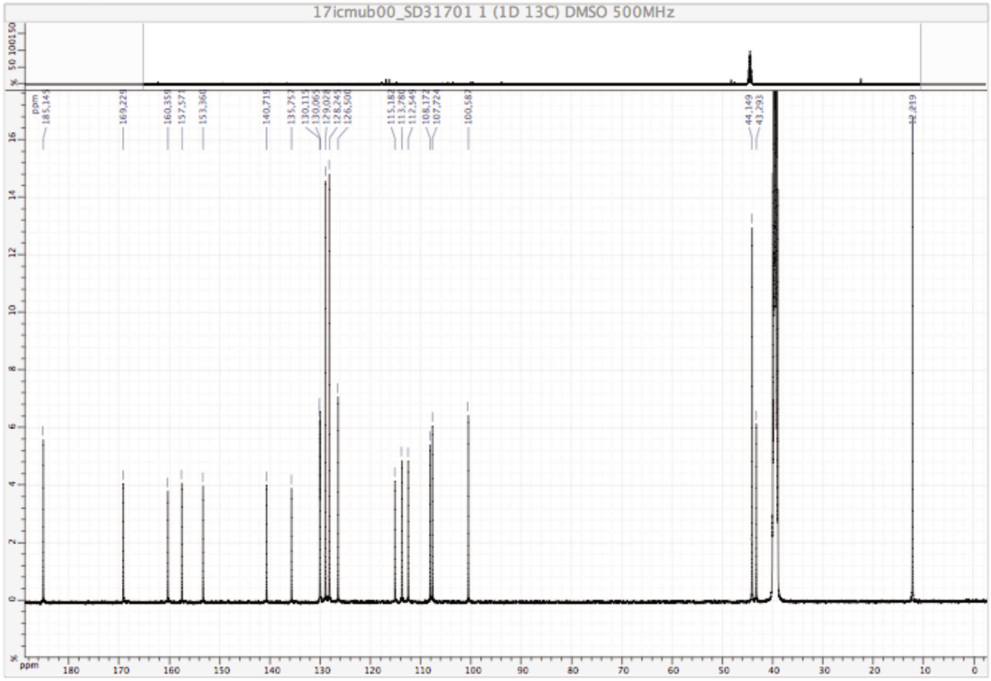
RP-HPLC elution profile of compound 3 (system A, detection at 260 nm)



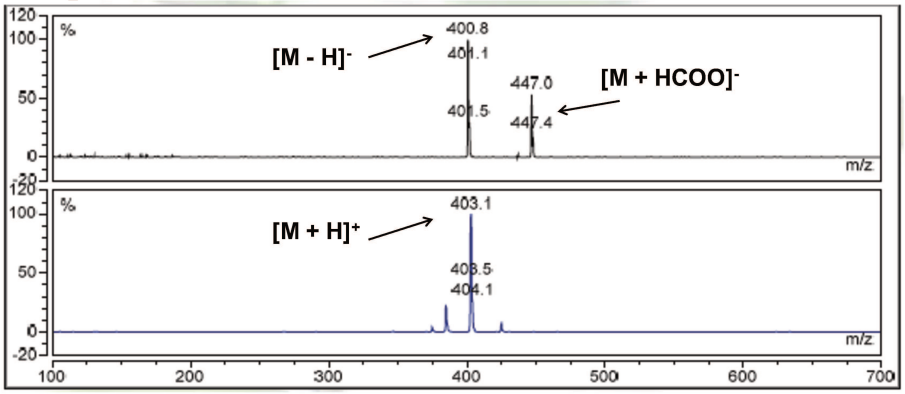
^{1}H NMR spectrum of compound 4 in DMSO-d_6 (300 MHz)

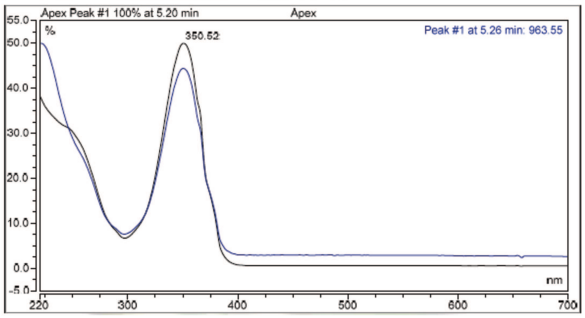


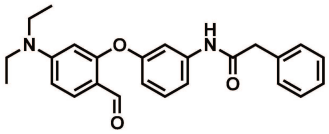
^{13}C NMR spectrum of compound 4 in DMSO-d_6 (126 MHz)



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

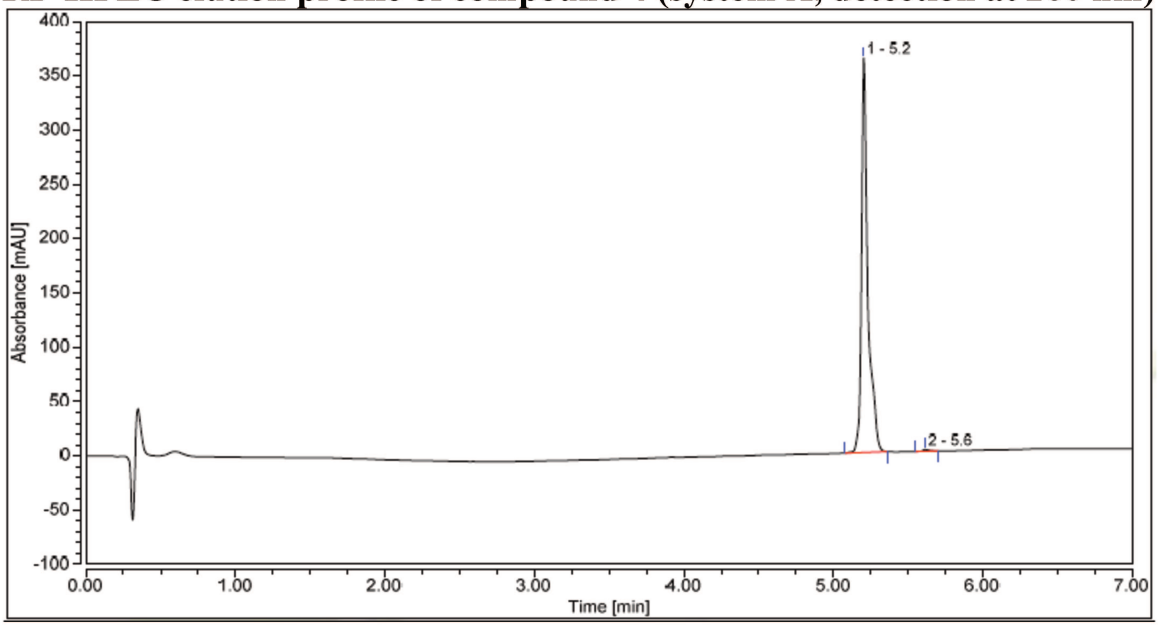




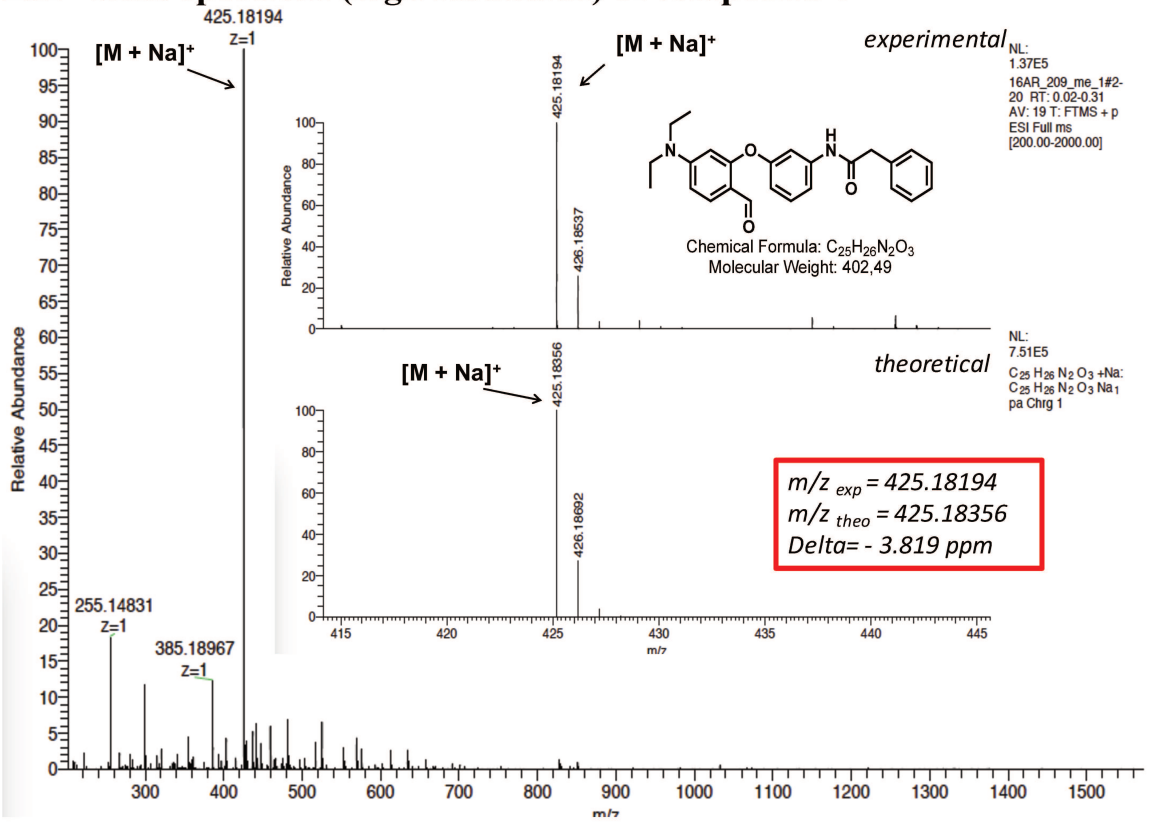


Chemical Formula: $C_{25}H_{26}N_2O_3$
Molecular Weight: 402,49

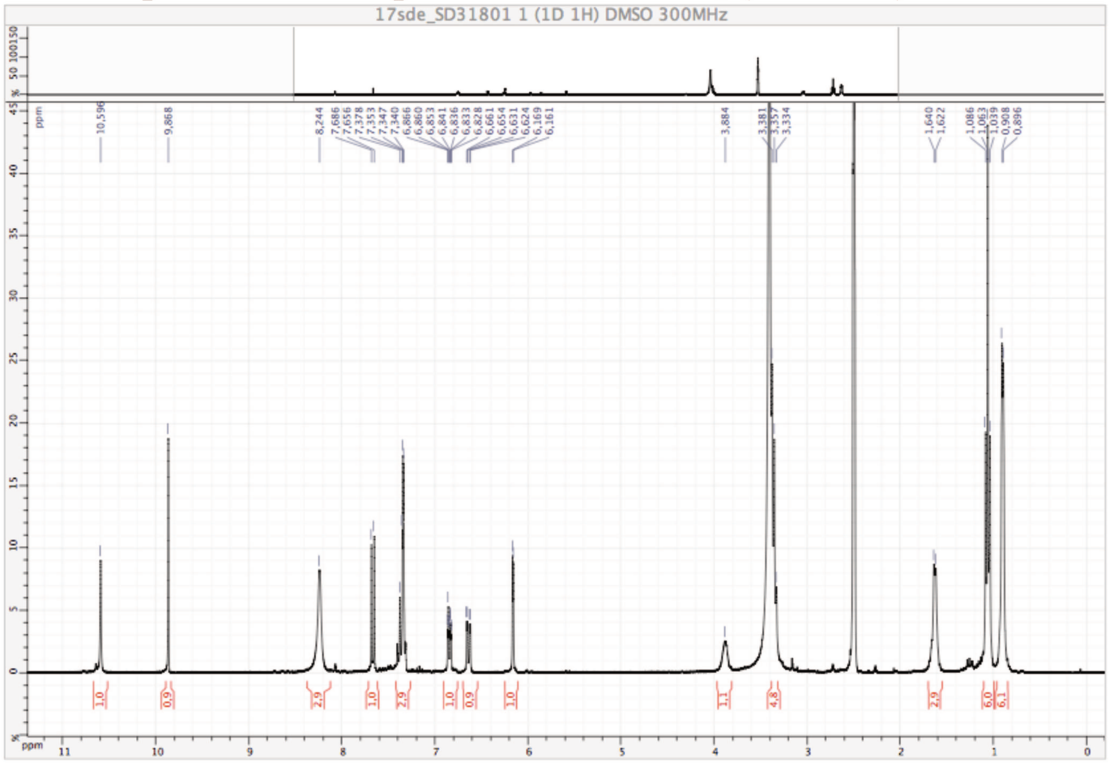
RP-HPLC elution profile of compound 4 (system A, detection at 260 nm)



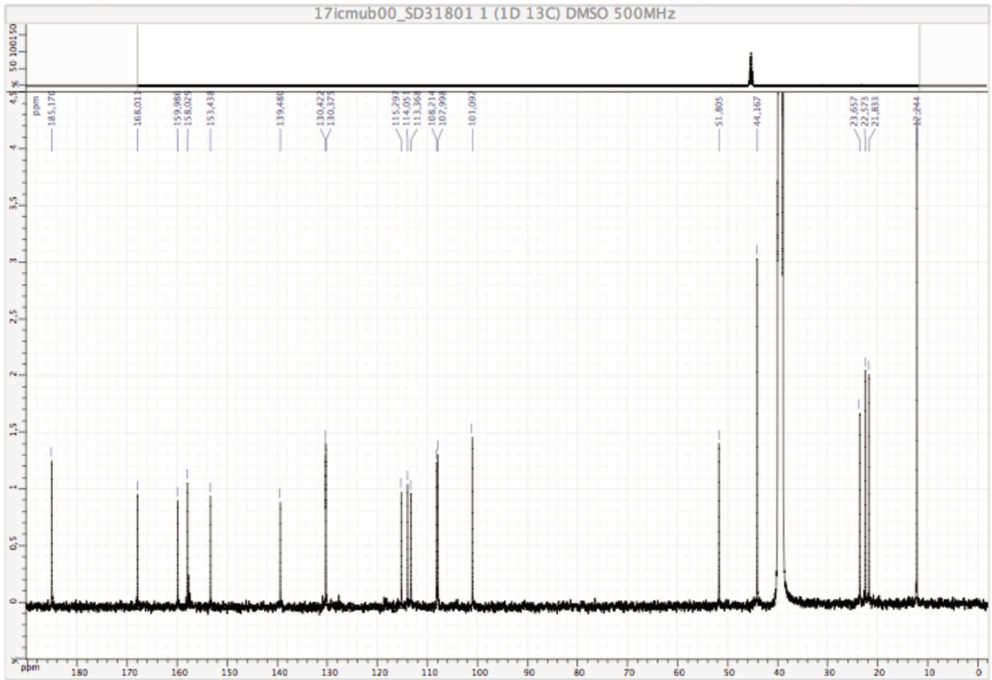
ESI+ mass spectrum (high resolution) of compound 4



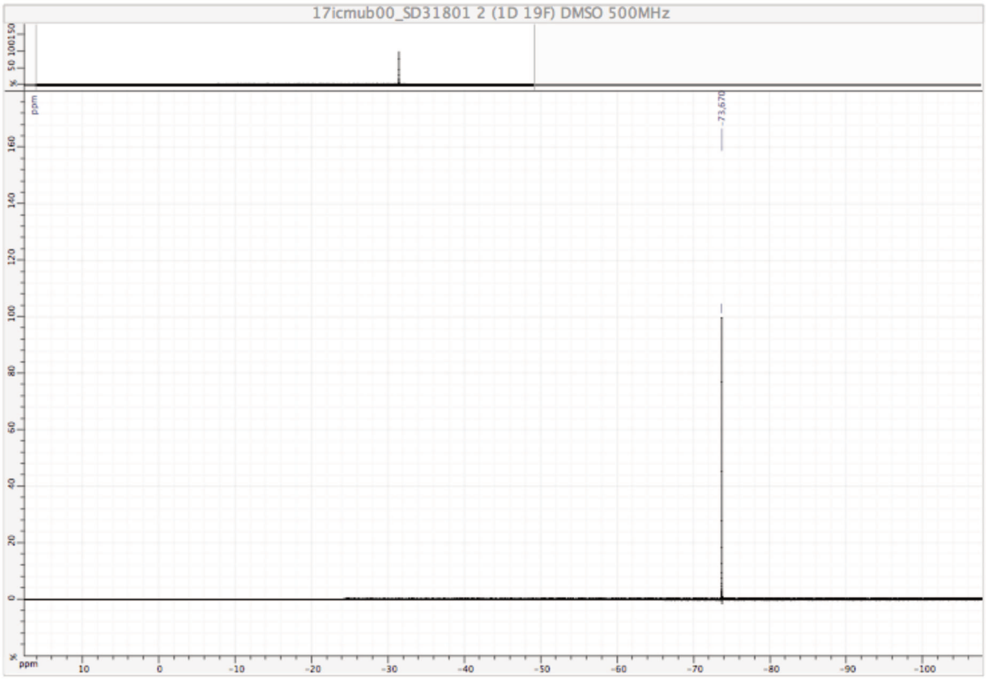
^{1}H NMR spectrum of compound 5 in DMSO-d_6 (300 MHz)



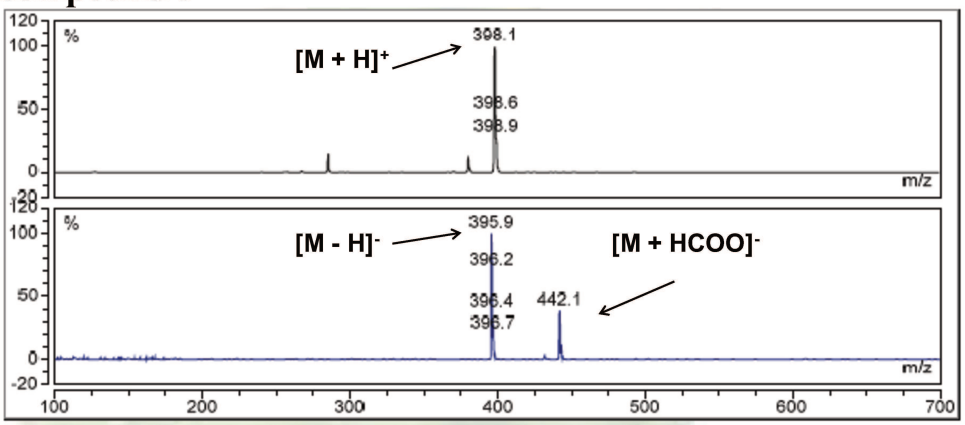
^{13}C NMR spectrum of compound 5 in DMSO-d_6 (126 MHz)

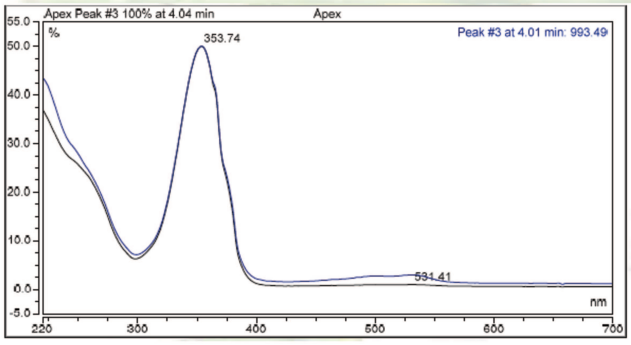


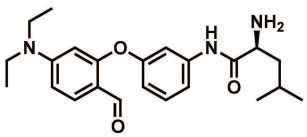
^{19}F NMR spectrum of compound 5 in DMSO-d_6 (470 MHz)



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

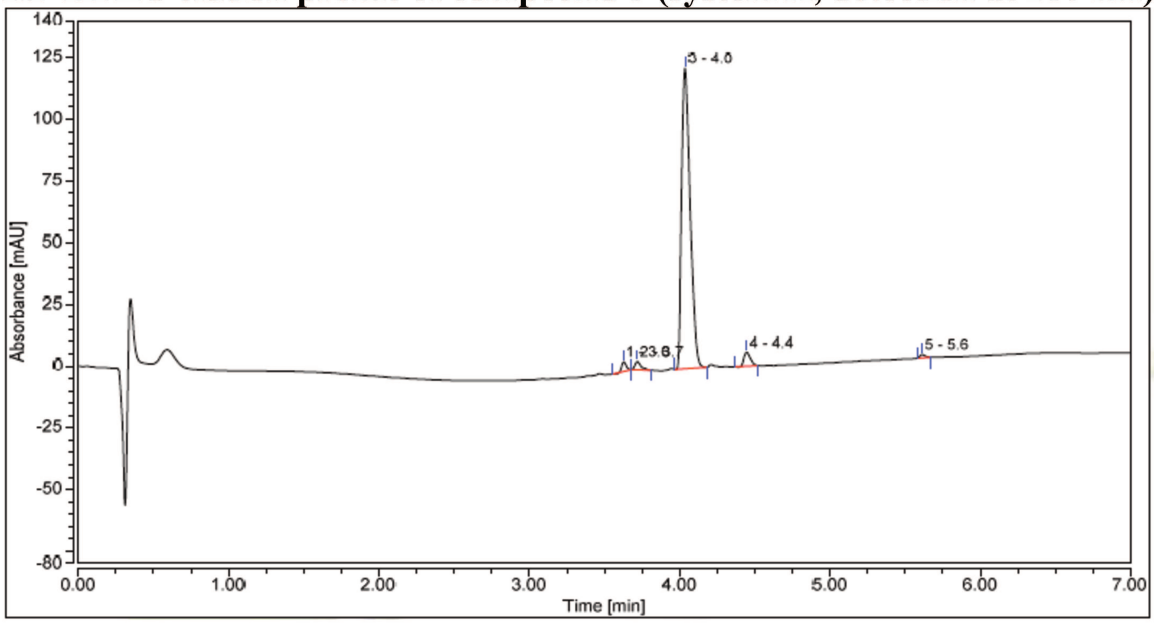




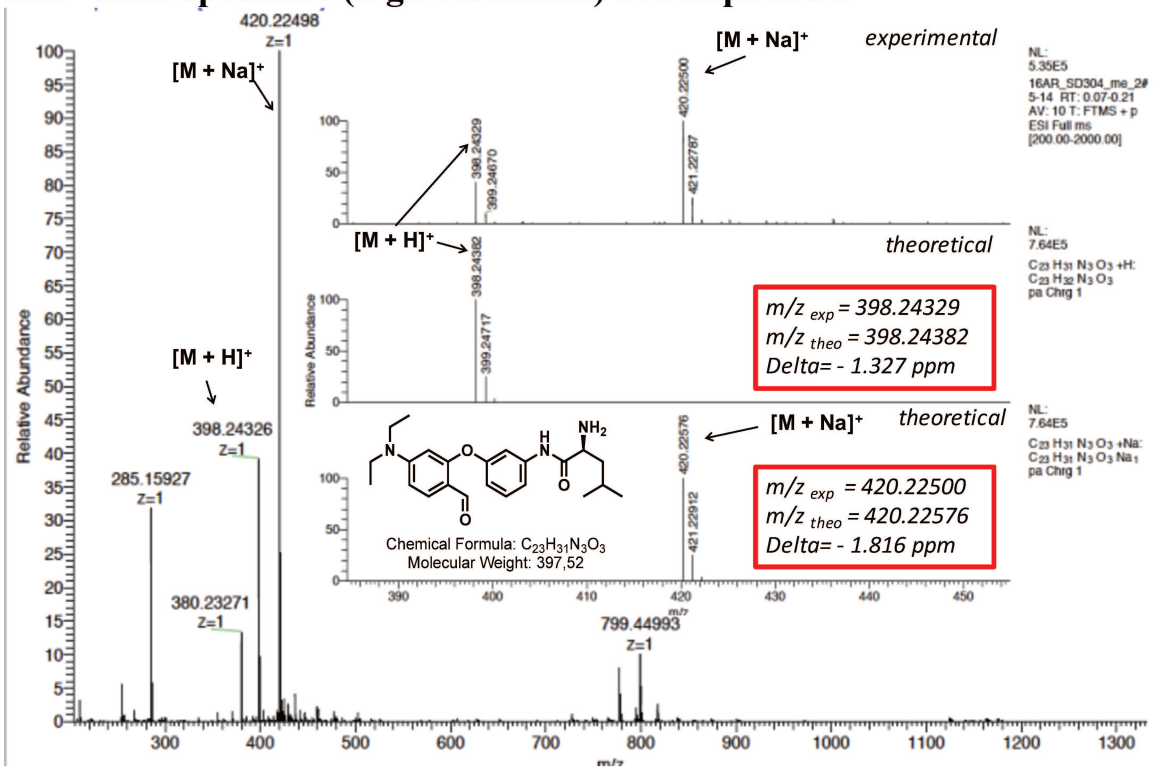


Chemical Formula: $C_{23}H_{31}N_3O_3$
Molecular Weight: 397,52

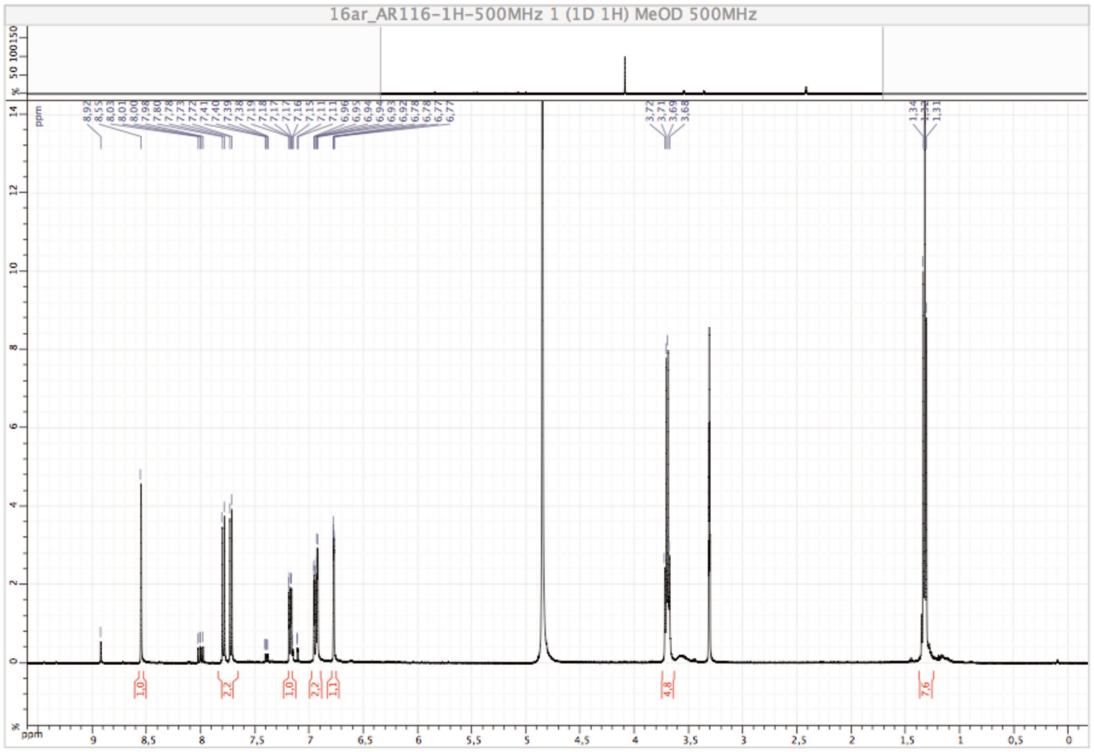
RP-HPLC elution profile of compound 5 (system A, detection at 260 nm)



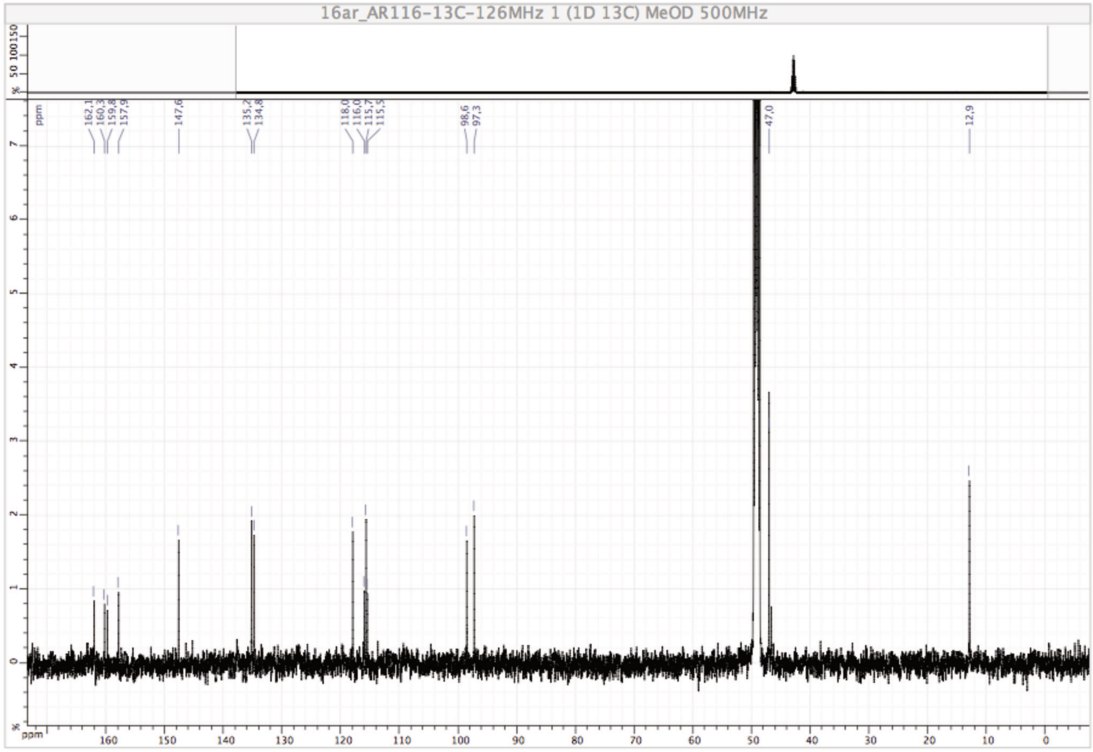
ESI+ mass spectrum (high resolution) of compound 5



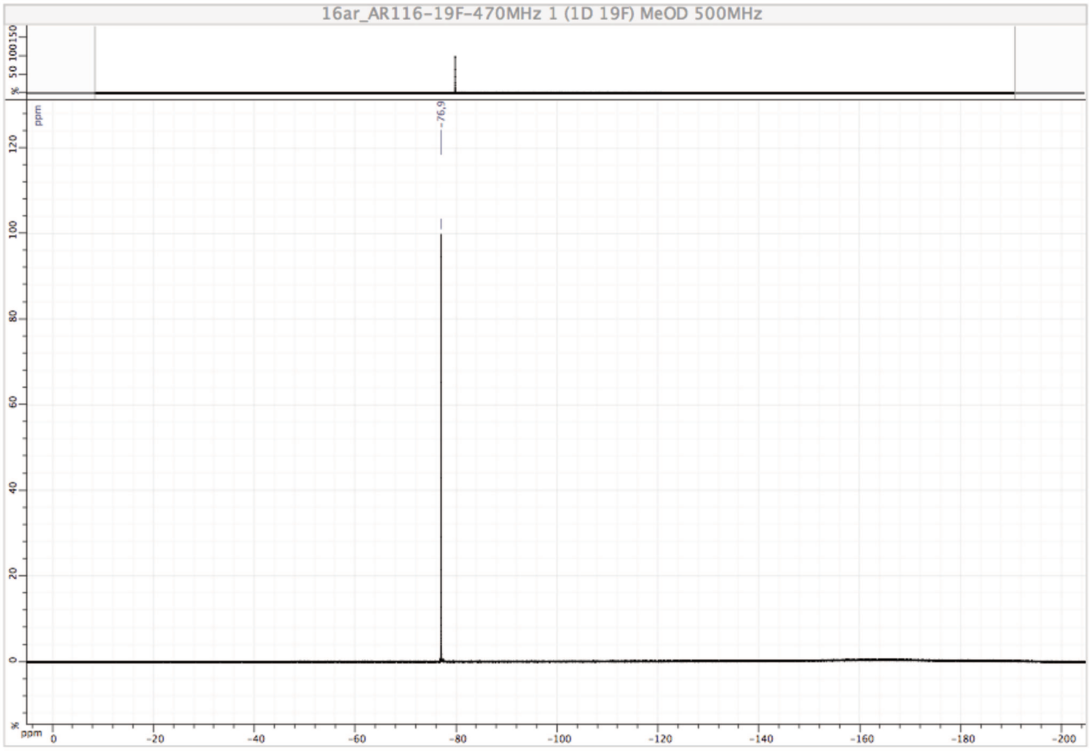
^{1}H NMR spectrum of 6 in CD_3OD (500 MHz)



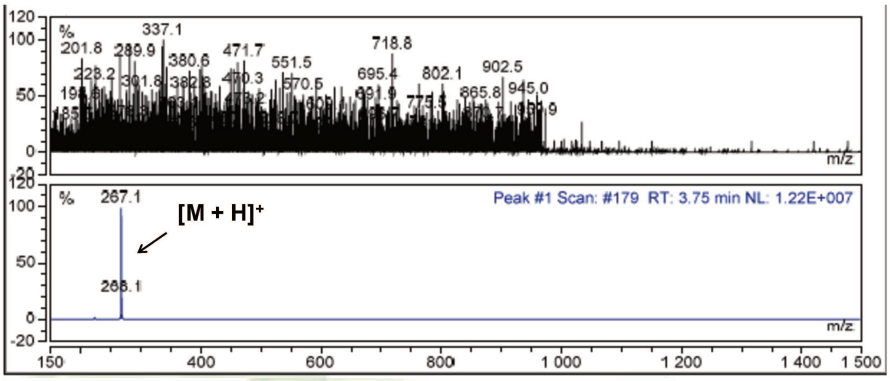
^{13}C NMR spectrum of 6 in CD_3OD (126 MHz)

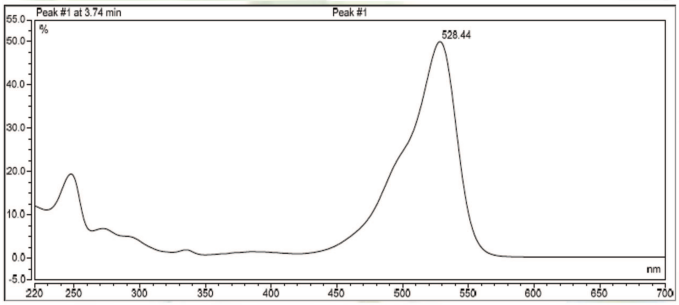


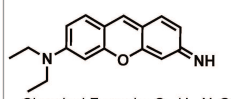
^{19}F NMR spectrum of 6 in CD_3OD (470 MHz)



ESI+ / ESI- mass spectrum (low resolution) and UV-vis spectrum of compound 6

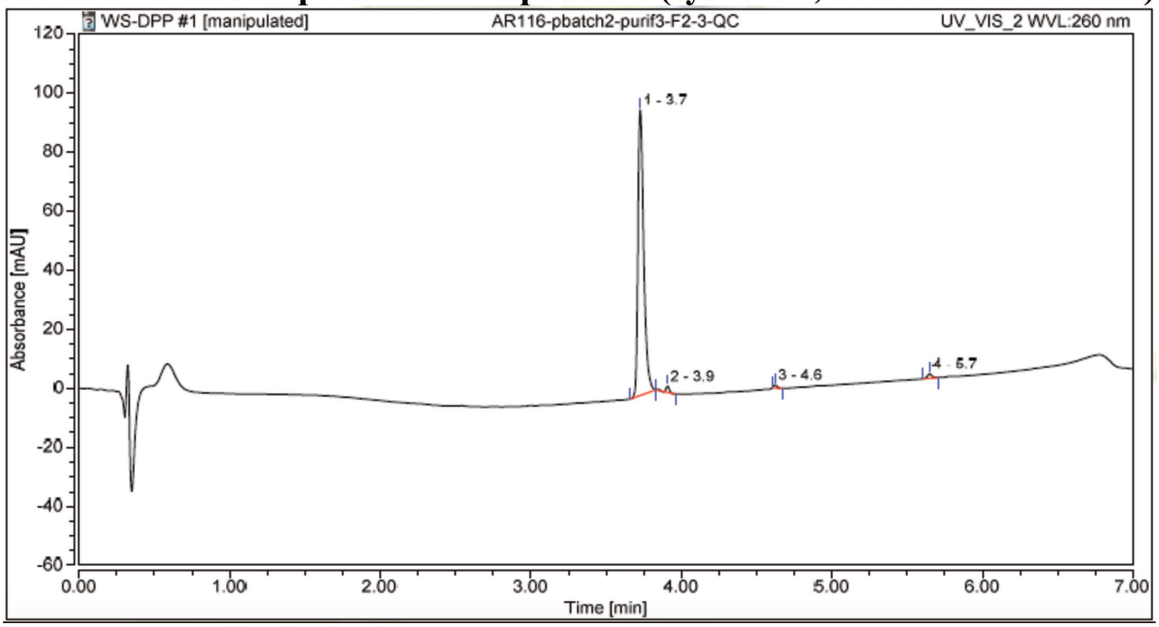






Chemical Formula: $C_{17}H_{16}N_2O$
Molecular Weight: 266,34

RP-HPLC elution profile of compound 6 (system B, detection at 260 nm)



RP-HPLC elution profile of compound 6 (system B, detection at 500 nm)

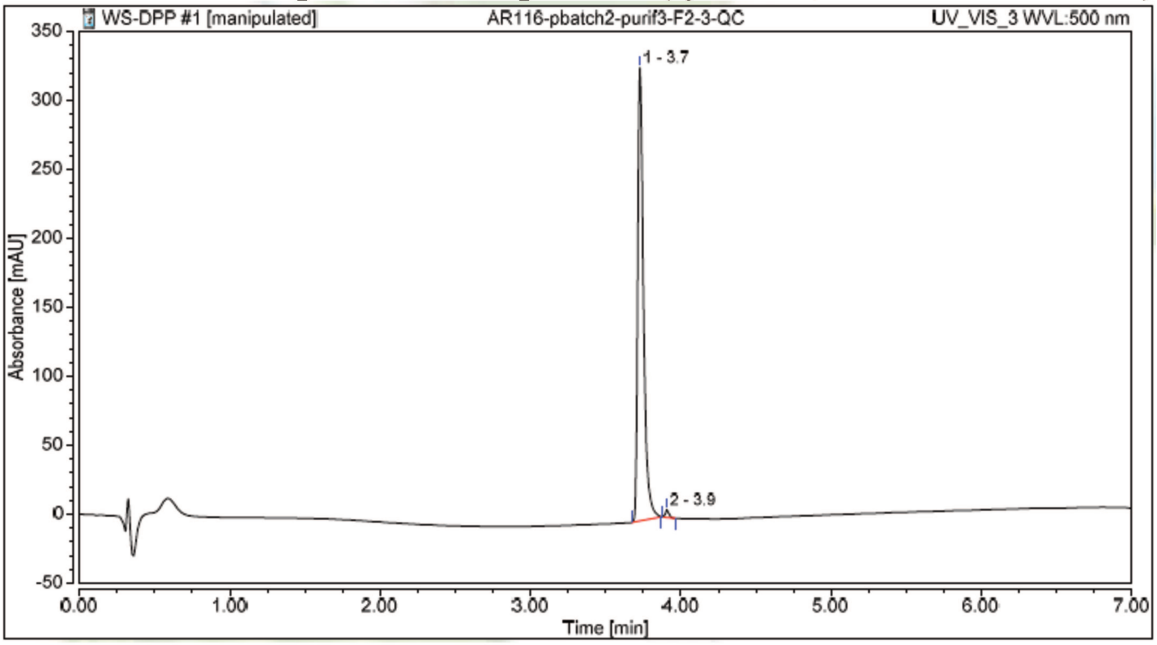


Fig. S1 Normalised UV-vis absorption spectra of fluorogenic probes 4 and 5 in PB (+ 0.3% CH_3CN and 0.15% CH_3CN respectively) at 25 °C.

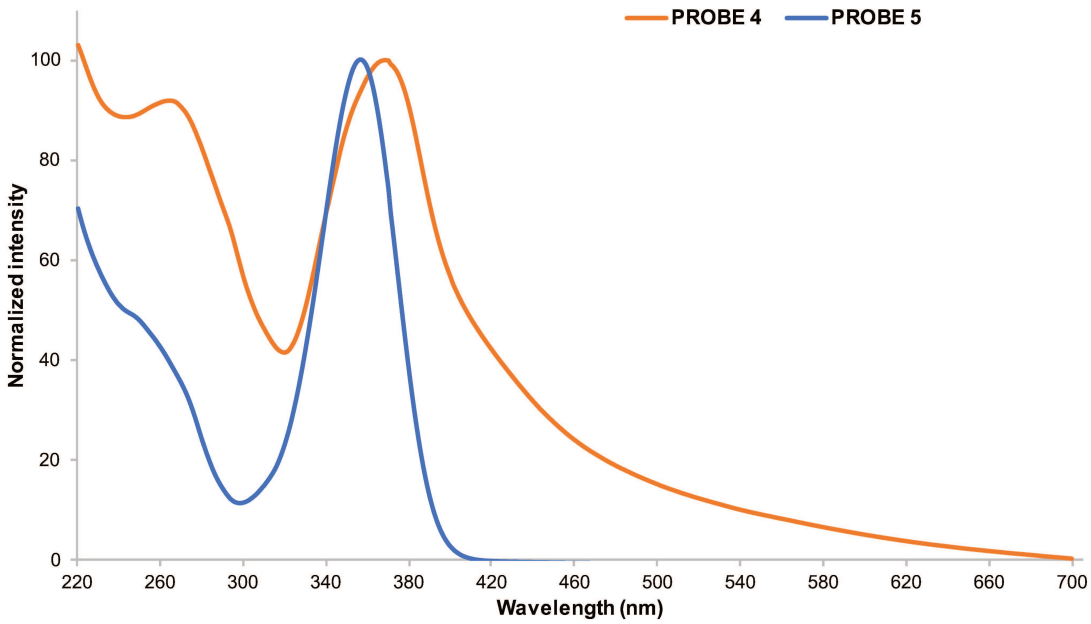


Fig. S2 Normalised absorption, excitation (Em. 545 nm) and emission (Ex. 460 nm) spectra of the pyronin 6 in PB at 25 °C.

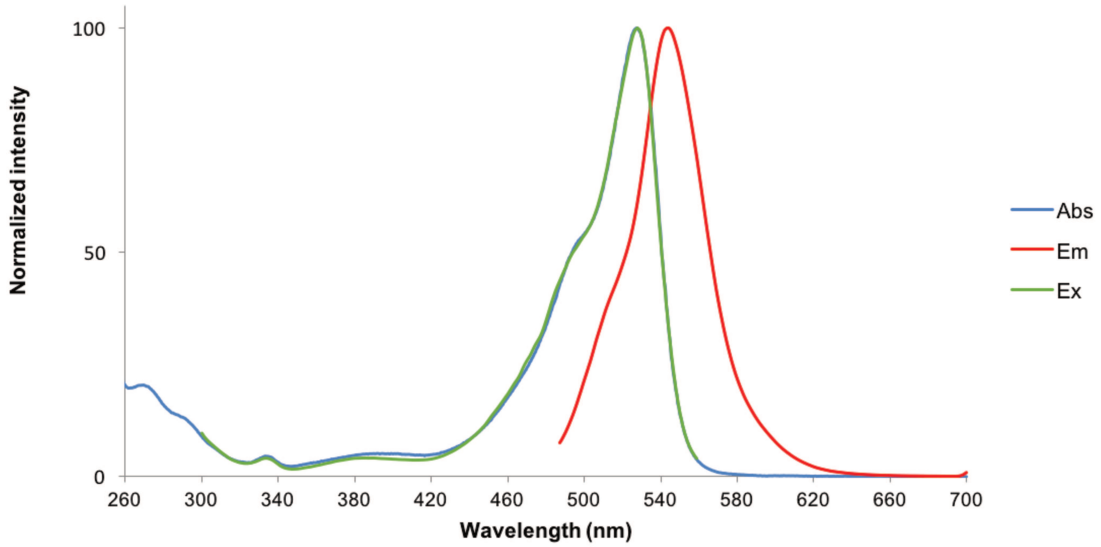


Fig. S3 Overlayed fluorescence emission spectra (Ex. 460 nm) of PGA-fluorogenic probe 4, LAP-fluorogenic probe 5 and pyronin 6 in PB at 25 °C (concentration: 1 μM)

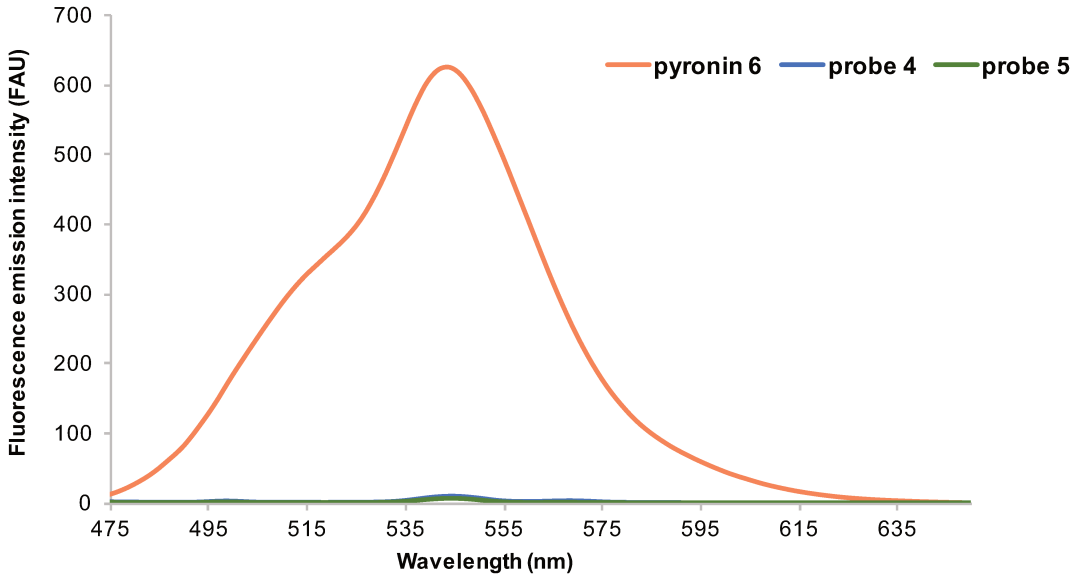


Fig. S4 Time-dependant changes in the green-yellow fluorescence intensity (Ex. 525 nm / Em. 545 nm, bandwidth 5 nm) of fluorogenic probe 4 (concentration: 1μM) in the presence of PGA (1 U) in various aqueous buffers at 37 °C

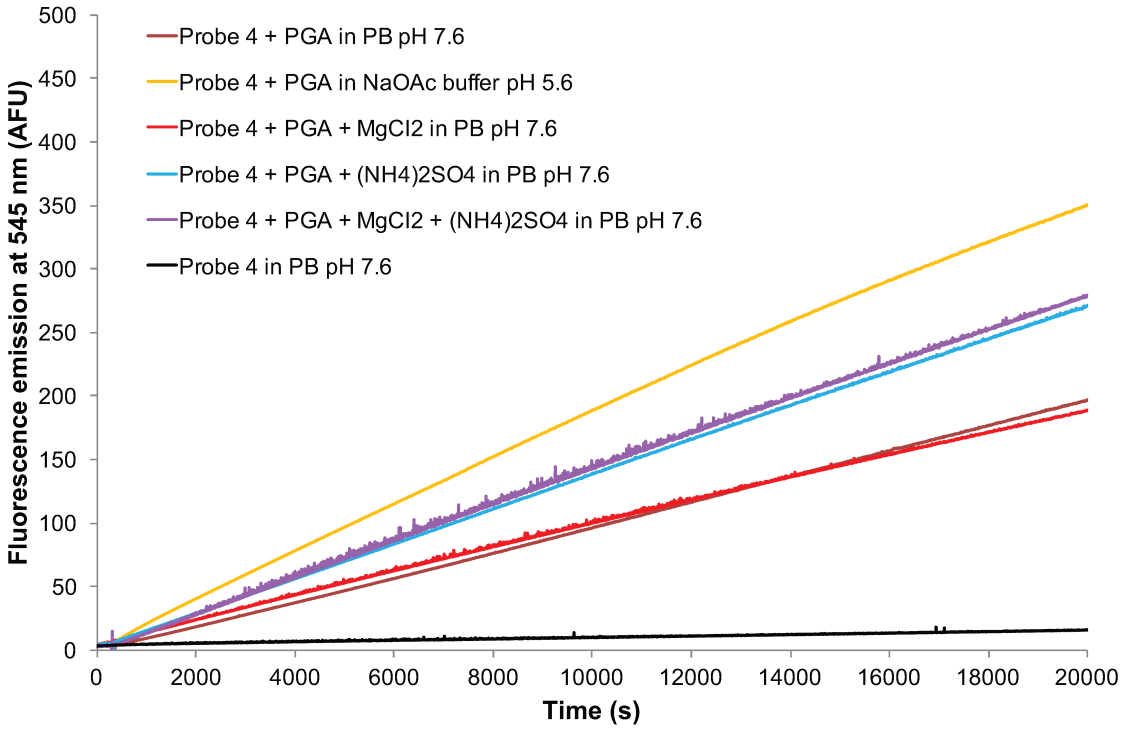


Fig. S5 Time-dependant changes in the green-yellow fluorescence intensity (Ex. 525 nm / Em. 545 nm, bandwidth 5 nm) of fluorogenic probe 4 in aqueous buffers at 37 °C (blanks)

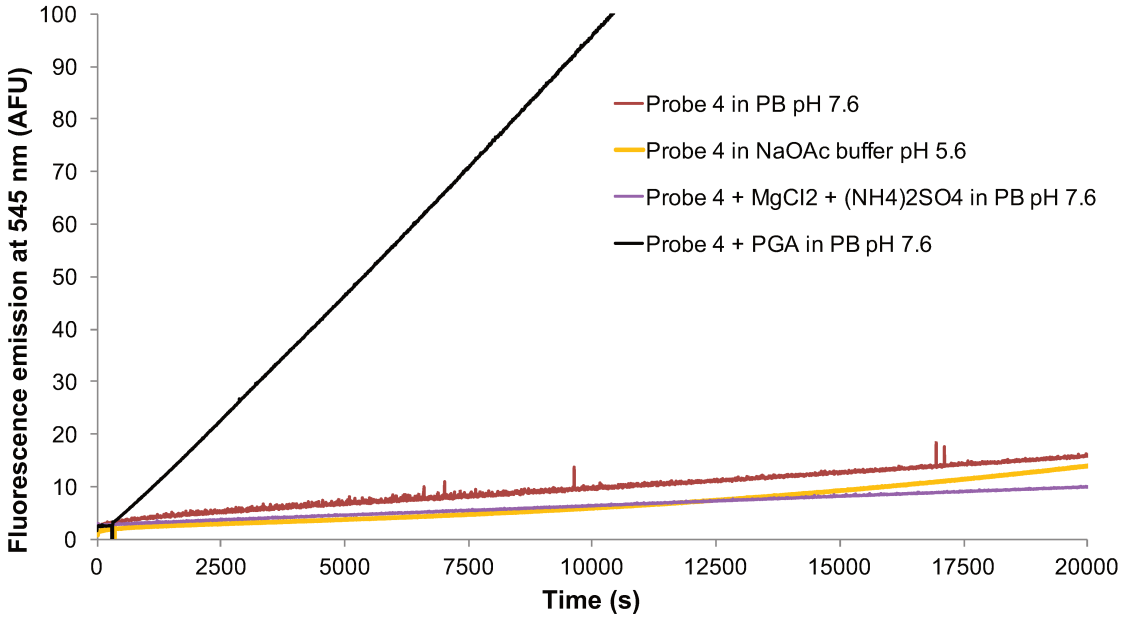


Fig. S6 Time-dependant changes in the green-yellow fluorescence intensity (Ex. 525 nm / Em. 545 nm, bandwidth 5 nm) of fluorogenic probe 5 in the presence of LAP (2 x 10^{-3} U) in various aqueous buffers at 37 °C

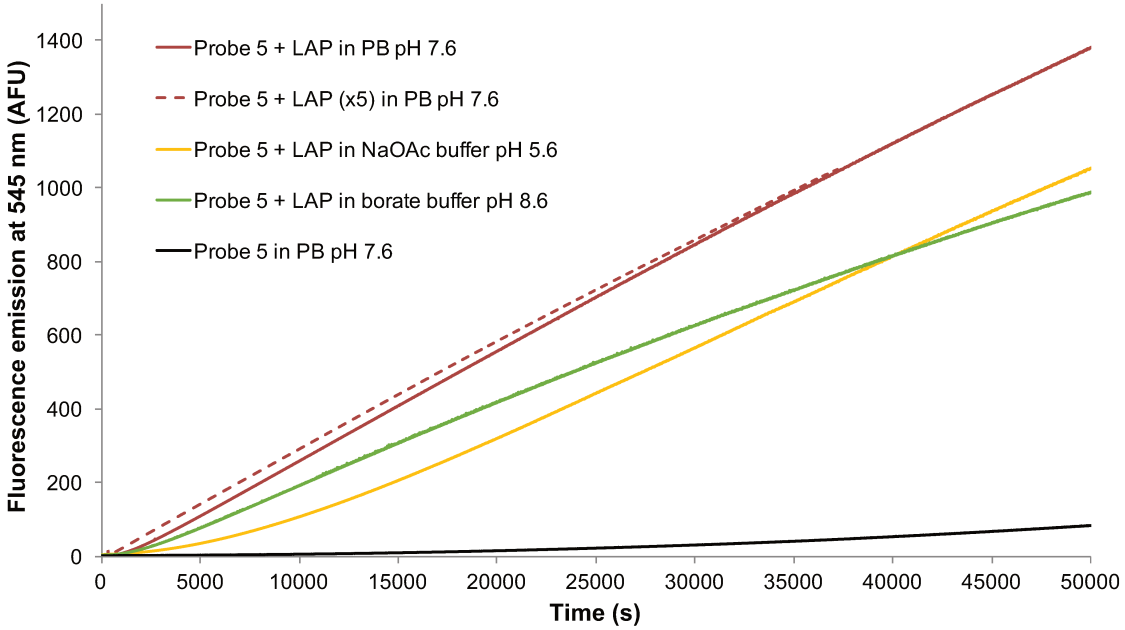


Fig. S7 Time-dependant changes in the green-yellow fluorescence intensity (Ex. 525 nm / Em. 545 nm, bandwidth 5 nm) of fluorogenic probe 5 in aqueous buffers at 37 °C (blanks)

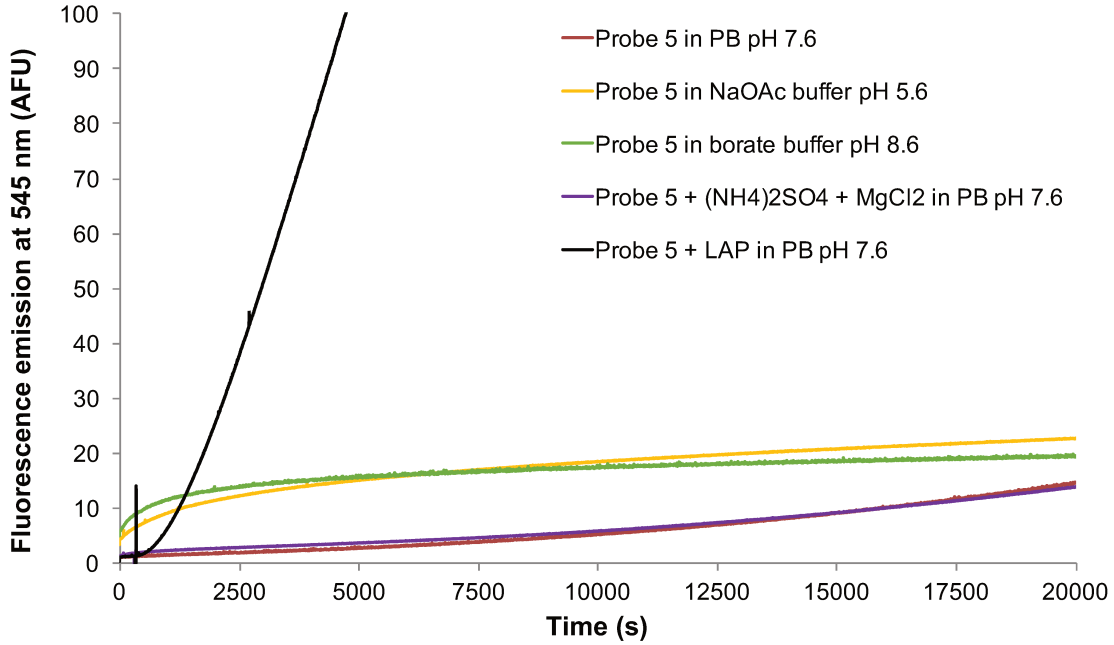


Fig. S8 Overlayed fluorescence emission spectra (Ex. 460 nm, bandwidth 5 nm) of enzymatic reaction mixtures (probe 5) after 24 h of incubation at 37 °C and authentic sample of pyronin 6 at the same concentration

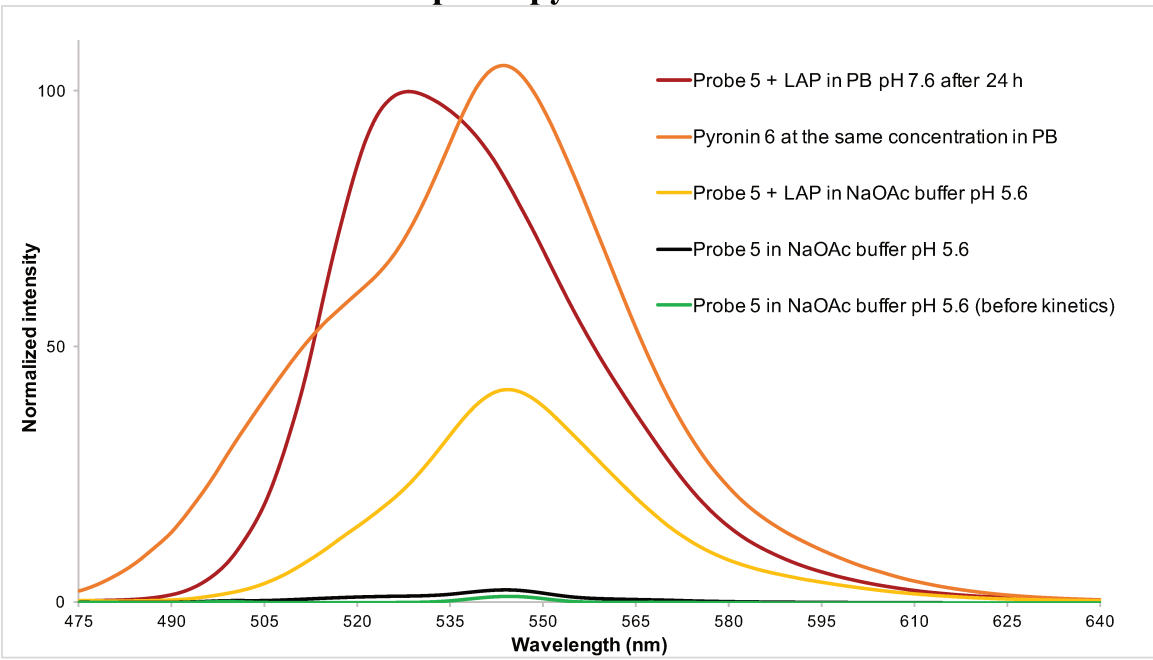


Fig. S9 Overlayed fluorescence emission spectra (Ex. 460 nm, bandwidth 5 nm) of enzymatic reaction mixtures (probe 4) after 24 h of incubation at 37 °C

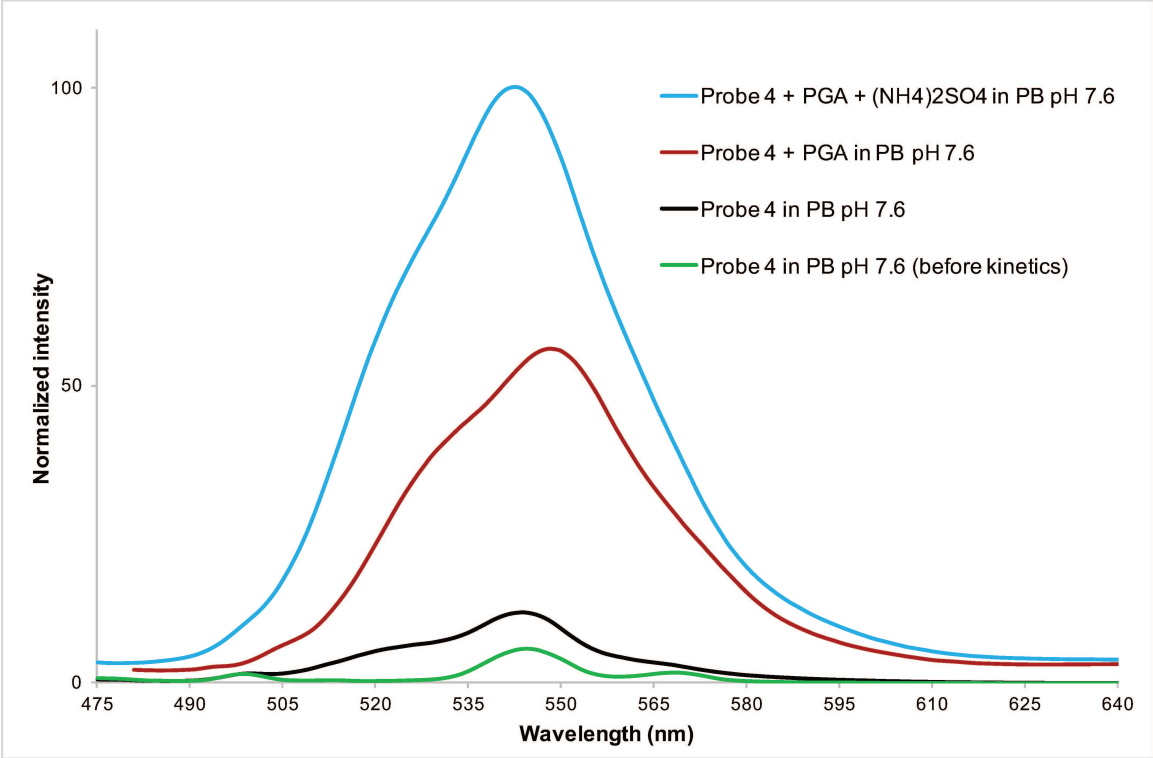
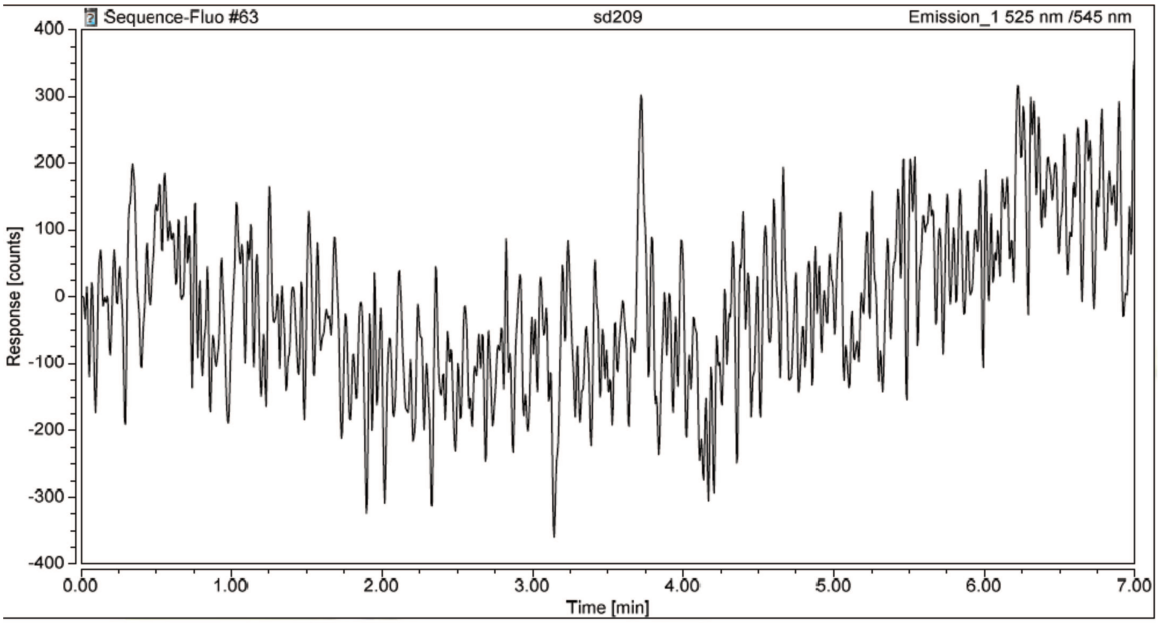


Fig. S10 RP-HPLC elution profiles (fluorescence detection, system H) of fluorogenic probes 4 and 5 before enzymatic activation



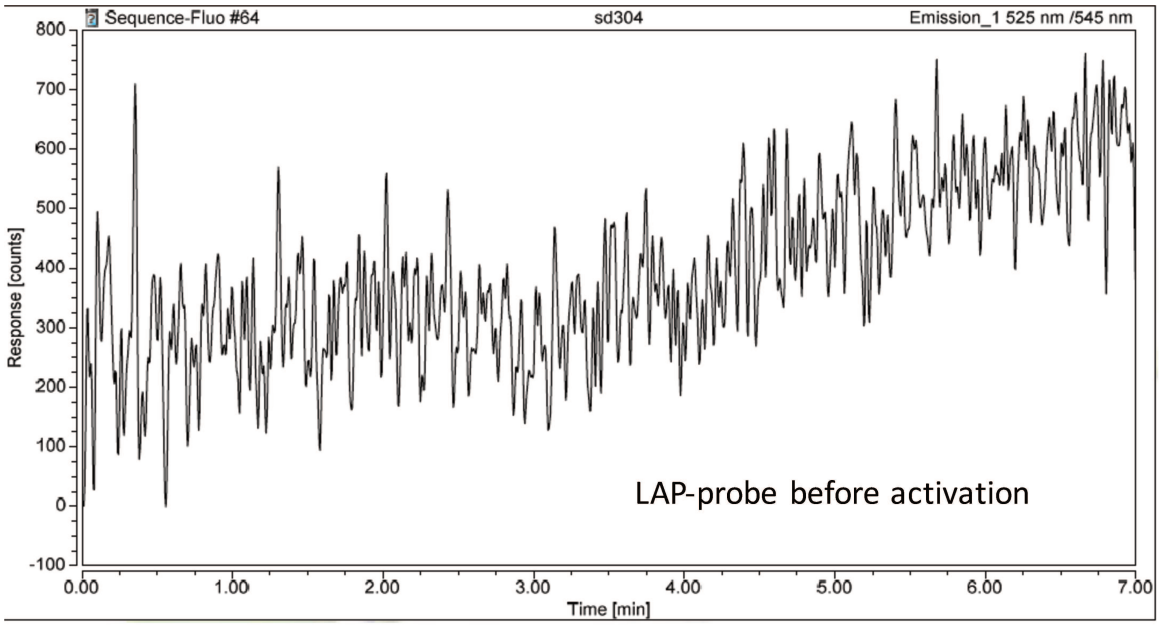
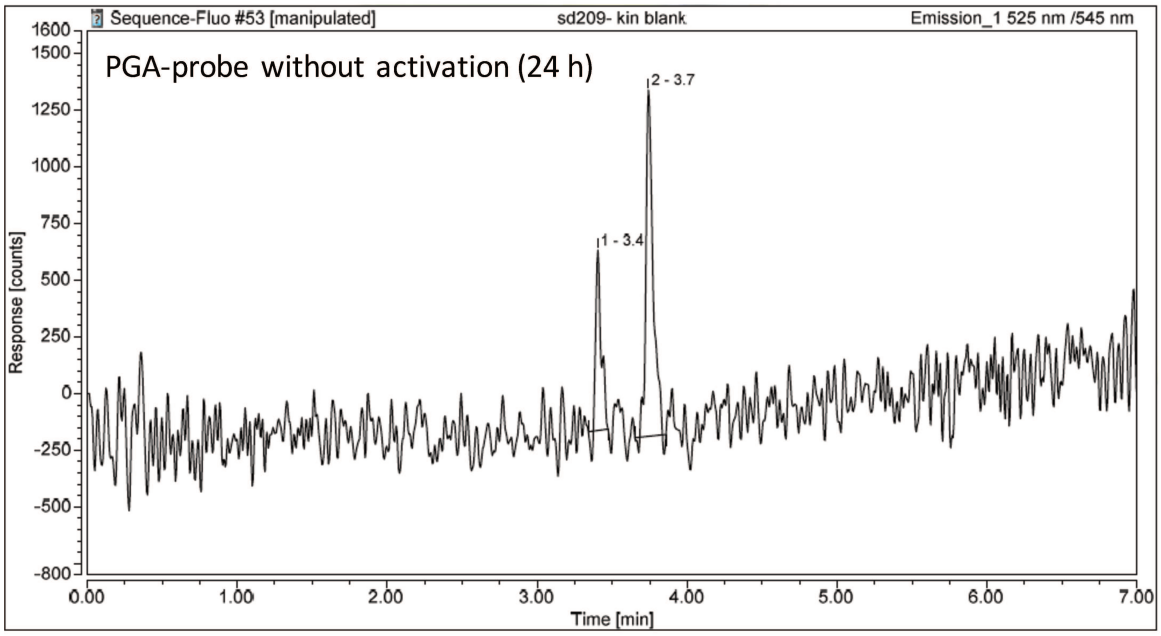


Fig. S11 RP-HPLC elution profiles (fluorescence detection, system H) of fluorogenic probes 4 and 5 without enzymatic activation (24 h of incubation)



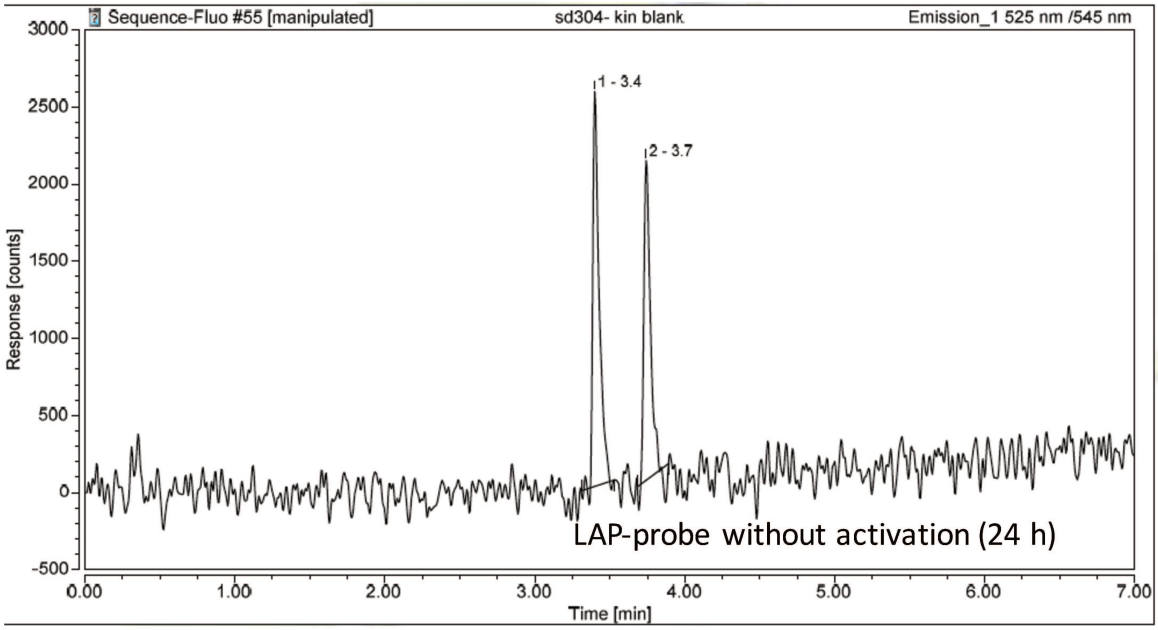
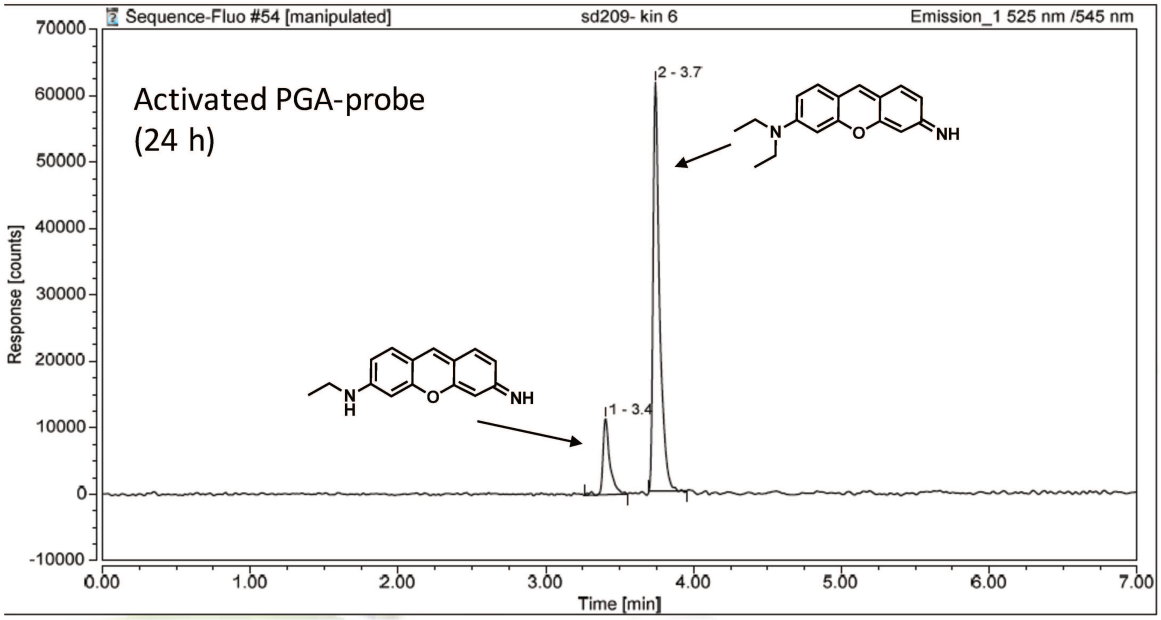
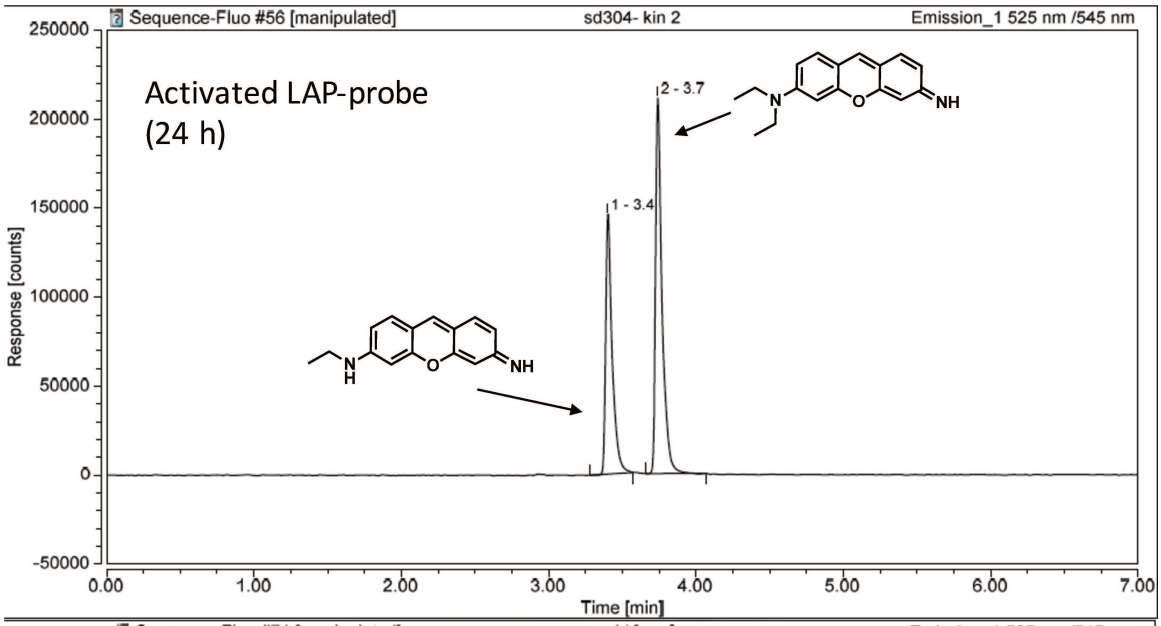


Fig. S12 RP-HPLC elution profiles (fluorescence detection, system H) of fluorogenic probes 4 and 5 after enzymatic activation (24 h) and reference 6




Sequence-Fluo #56 [manipulated]
sd304- kin 2
Emission_1 525 nm /545 nm
Activated LAP-probe
(24 h)
1 - 3.4
2 - 3.7
Response [counts]
Time [min]

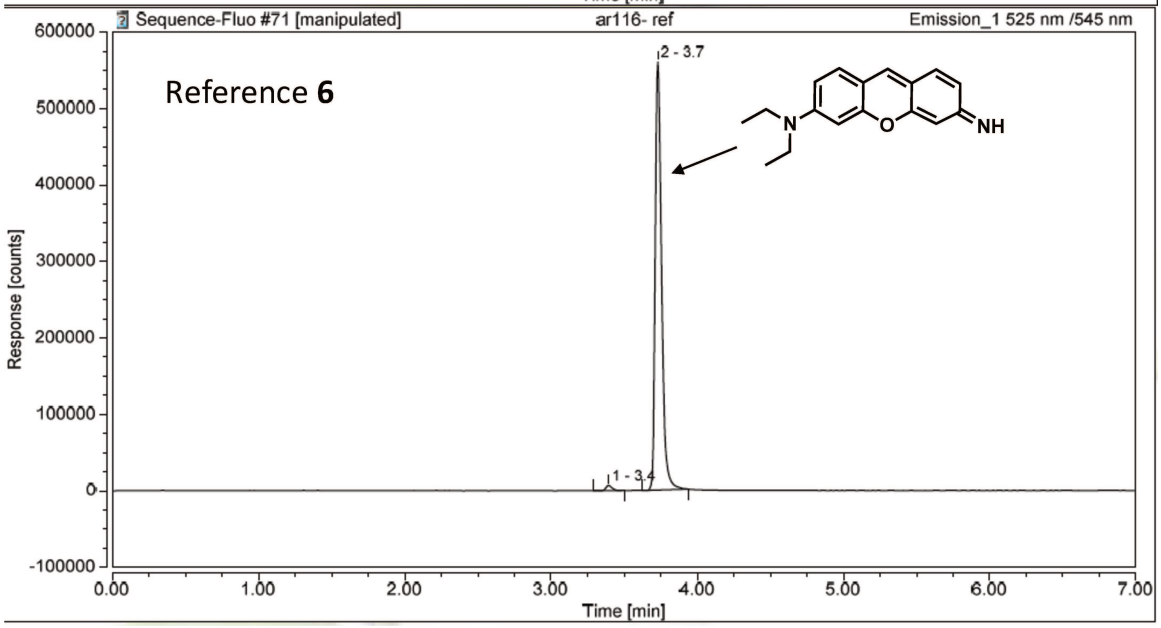

Sequence-Fluo #71 [manipulated]
ar116- ref
Emission_1 525 nm /545 nm
Reference 6
1 - 3.4
2 - 3.7
Response [counts]
Time [min]

Fig. S13 Time-dependant changes in the green-yellow fluorescence intensity (Ex. 525 nm / Em. 545 nm, bandwidth 5 nm) of fluorogenic probe 4 (concentration: 1 μM) in the presence of LAP (2 x 10^{-3} U) in PB at 37 °C (negative control)

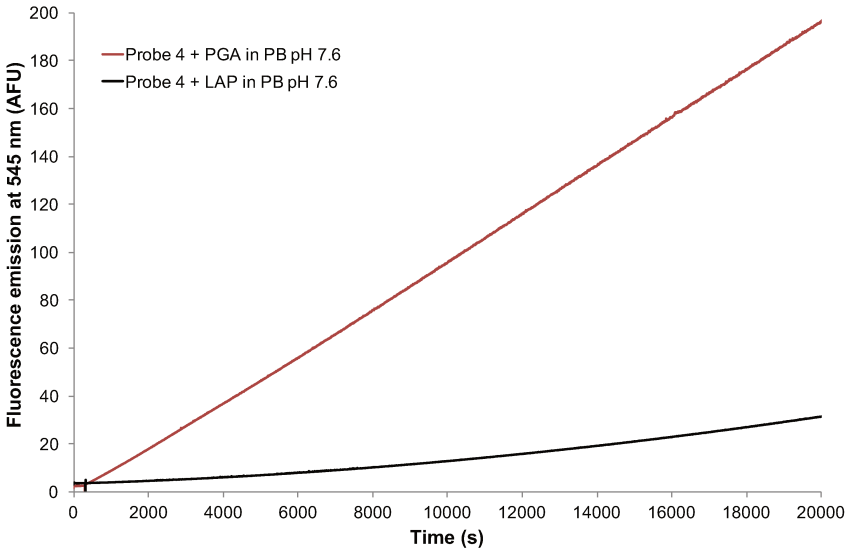


Fig. S14 Time-dependant changes in the green-yellow fluorescence intensity (Ex. 525 nm / Em. 545 nm, bandwidth 5 nm) of fluorogenic probe 5 (concentration: 1 μM) in the presence of PGA (1 U) in PB at 37 °C (negative control)

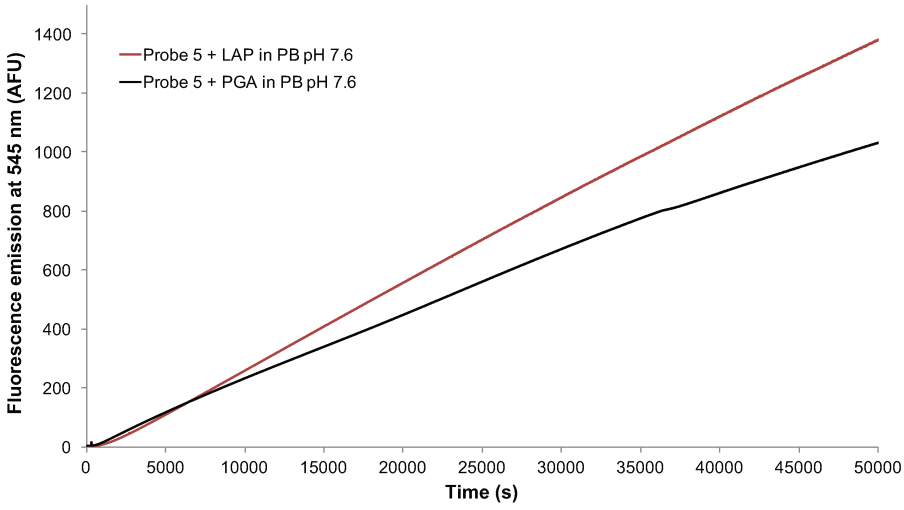


Fig. S15 RP-HPLC-MS analyses - Identification of "relevant" molecules/ intermediates formed during the enzymatic activation of probes 4 and 5 (system G)

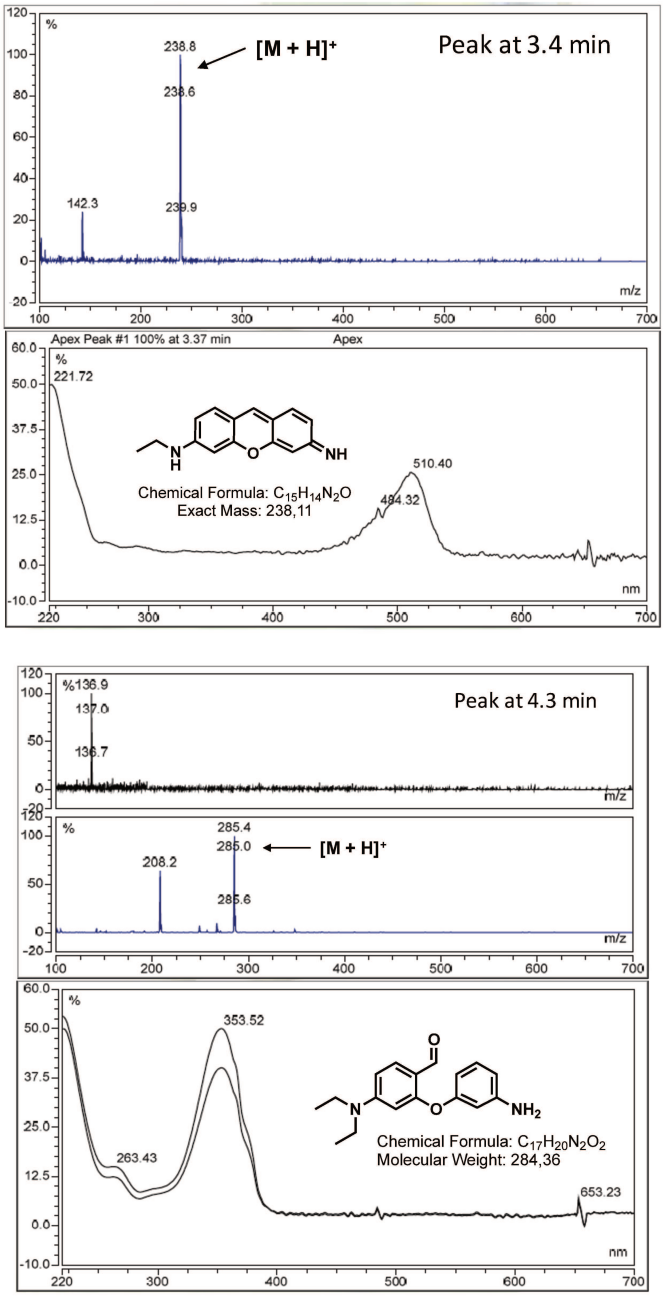
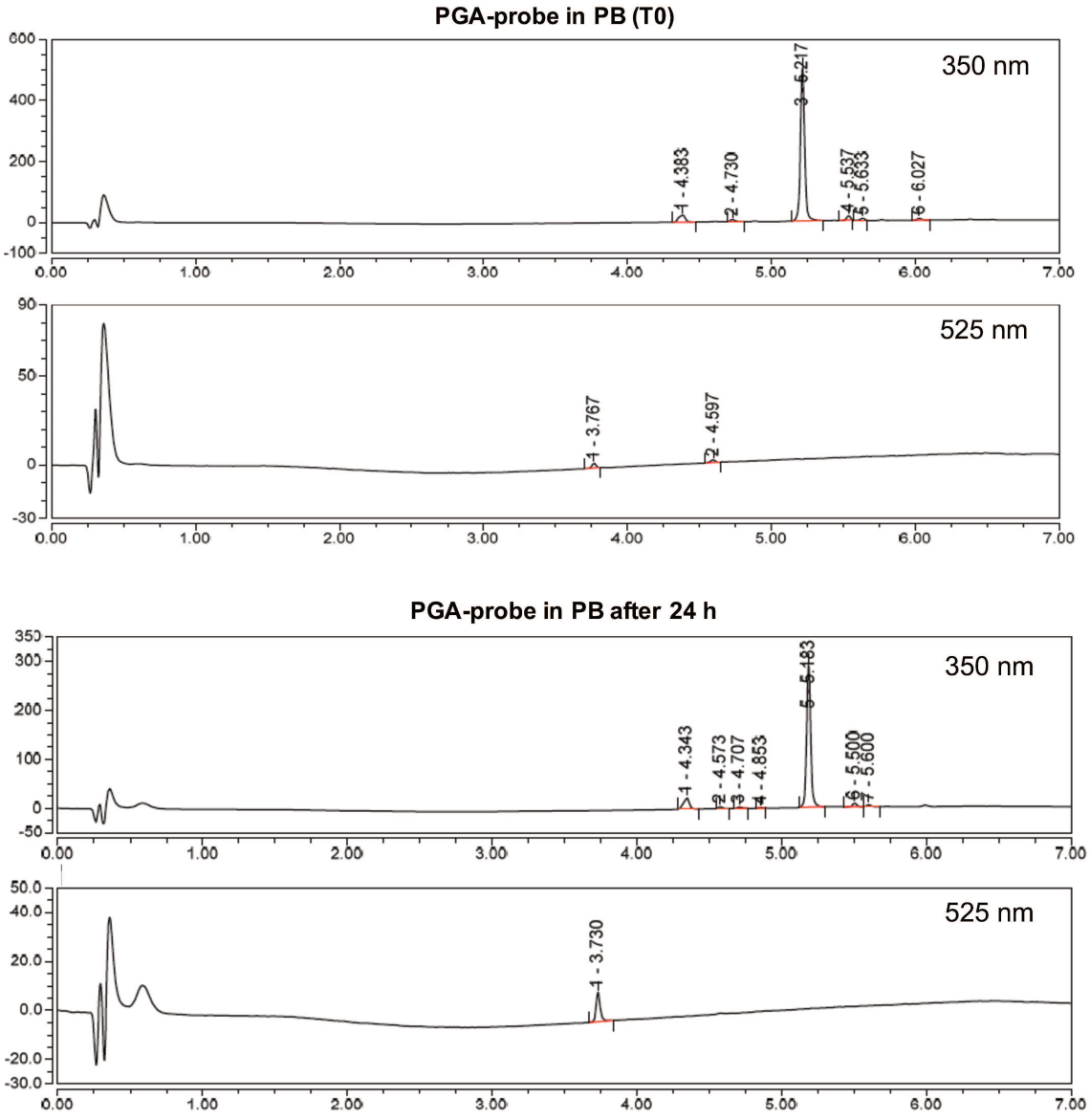
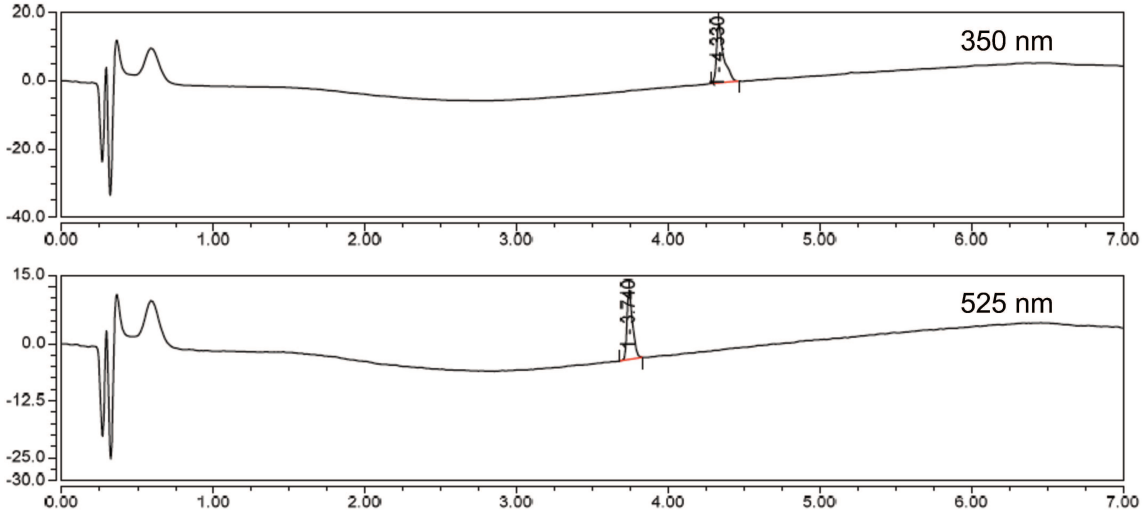


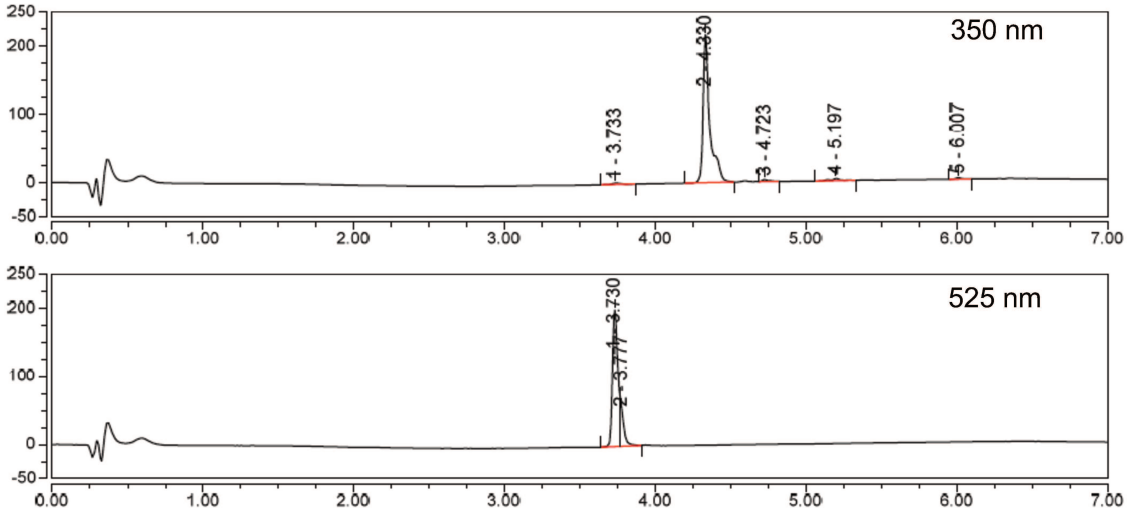
Fig. S16 RP-HPLC elution profiles (system G) of fluorogenic probe 4 after incubation in PB alone (0 min and after 24 h) or with enzyme (PGA: 2 h, 5 h, 8 h and 24 h)



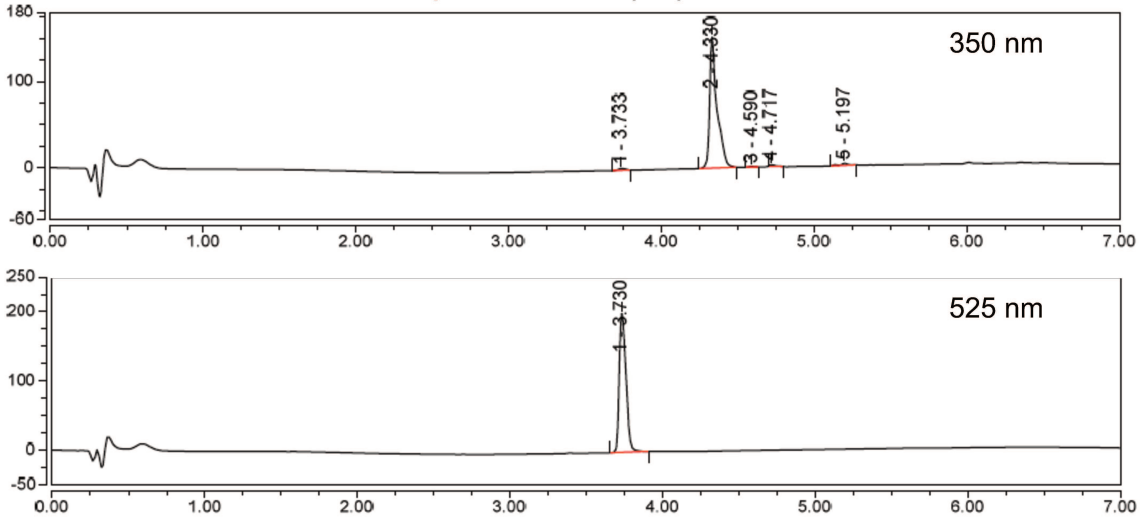
PGA-probe with PGA (1 U) after 2 h



PGA-probe with PGA (1 U) after 5 h



PGA-probe with PGA (1 U) after 8 h



PGA-probe with PGA (1 U) after 24 h

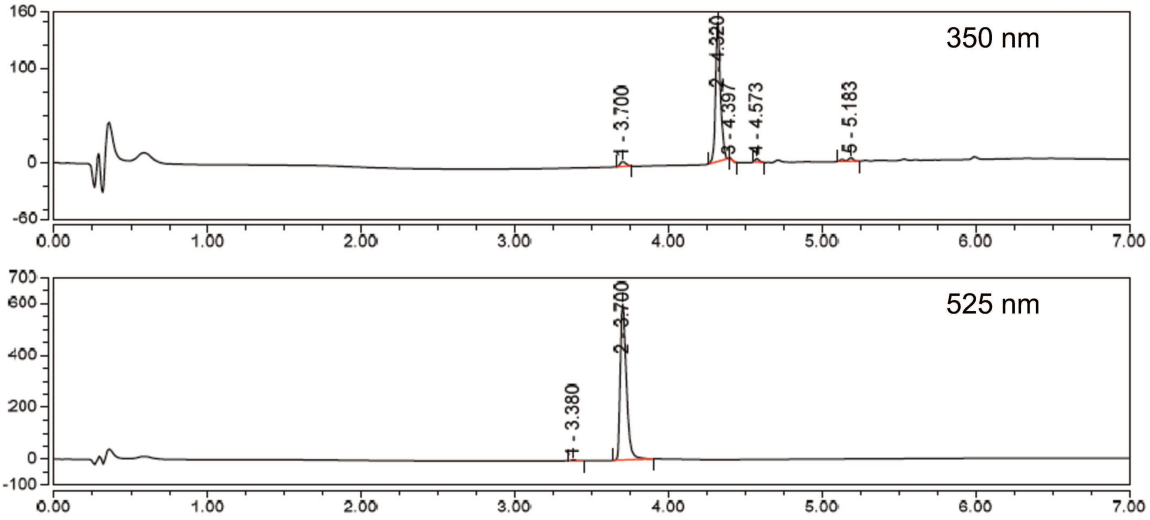
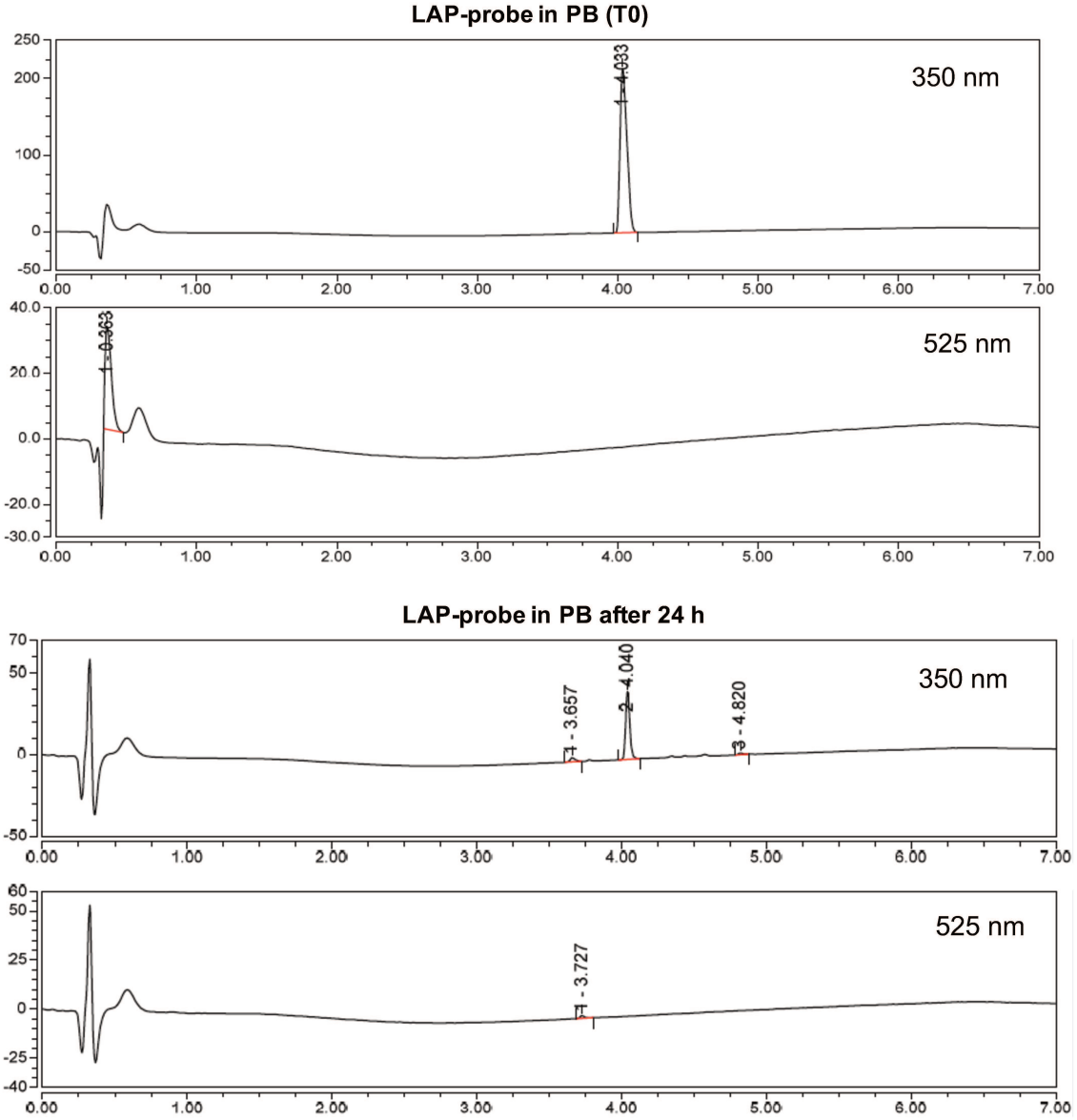
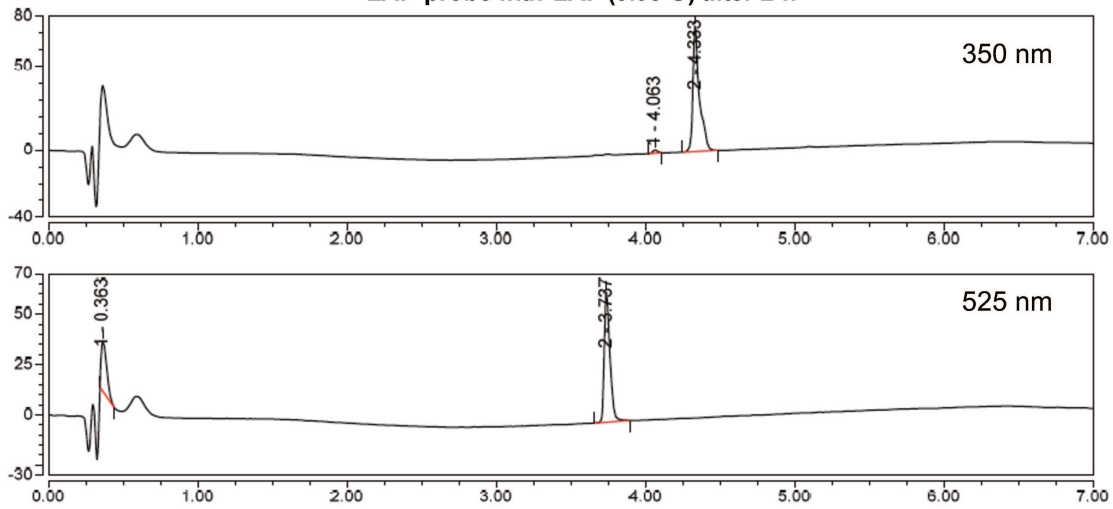


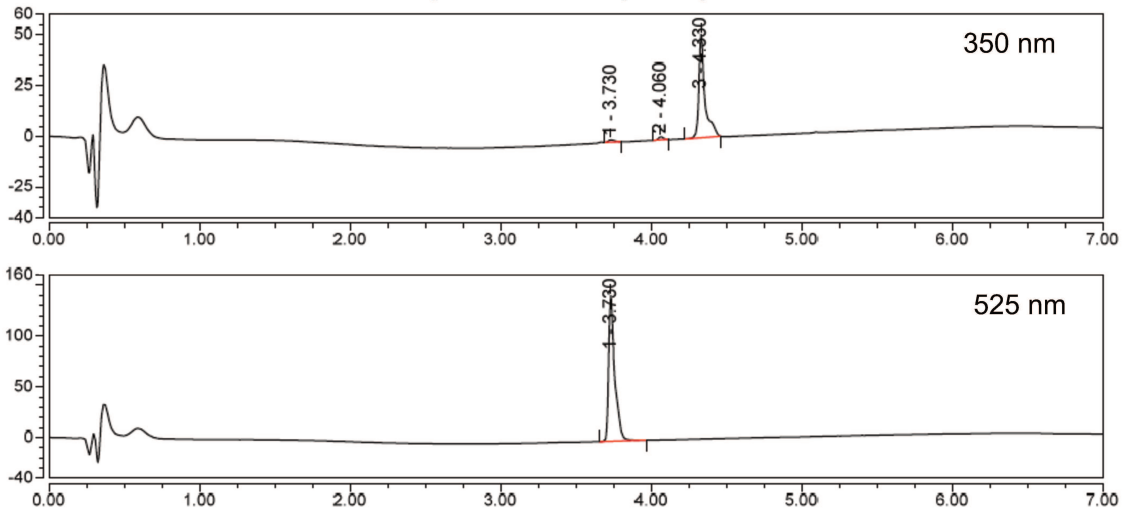
Fig. S17 RP-HPLC elution profiles (system G) of fluorogenic probe 5 after incubation in PB alone (0 min and after 24 h) or with enzyme (LAP: 2 h, 5 h, 8 h and 24 h)



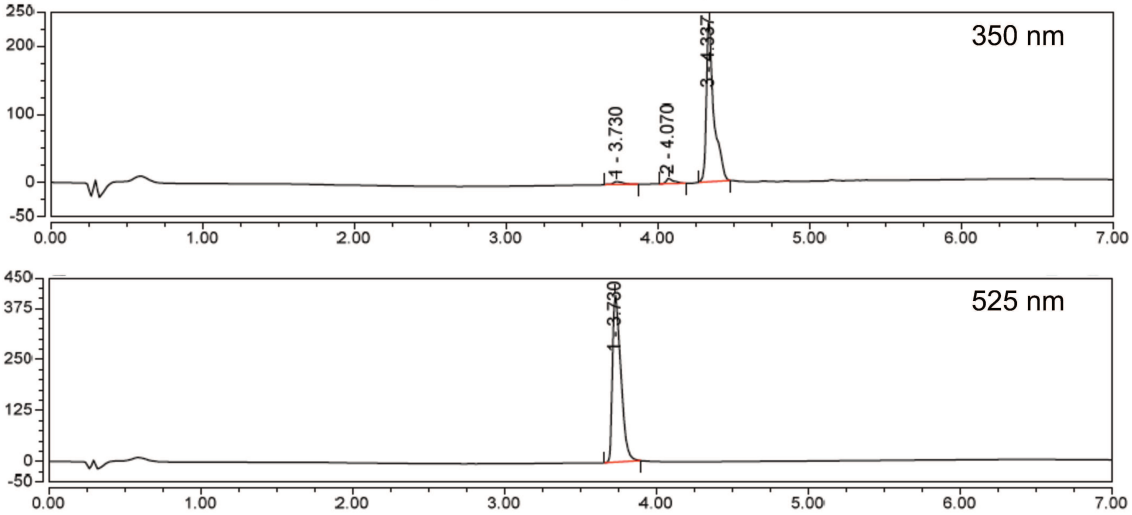
LAP-probe with LAP (0.05 U) after 2 h

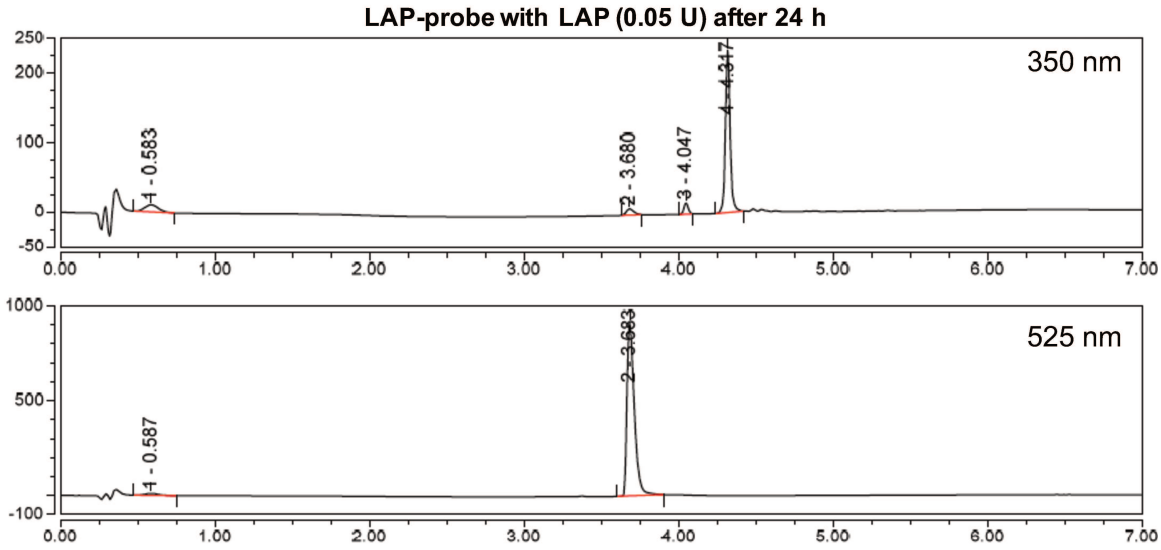


LAP-probe with LAP (0.05 U) after 5 h



LAP-probe with LAP (0.05 U) after 8 h




LAP-probe with LAP (0.05 U) after 24 h
350 nm
1 - 0.583
2 - 3.680
3 - 4.047
4 - 4.317
525 nm
1 - 0.587
2 - 3.683

SI.2.3. Experimental section and "Supporting Information" file of Article 3: Synthesis of N,N-Dialkylamino-nor-Dihydroxanthene-Hemicyanine Fused Near-Infrared Fluorophores and Their First Water-Soluble and/or Bioconjugatable Analogues

CHEMISTRY
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Supporting Information

Synthesis of *N*,*N*-Dialkylamino-*nor*-Dihydroxanthene-Hemicyanine Fused Near-Infrared Fluorophores and Their First Water-Soluble and/or Bioconjugatable Analogues

Michelle Jui Hsien Ong[+],[a] Sylvain Debieu[+],[b] Mathieu Moreau,[b] Anthony Romieu,*[b, c] and Jean-Alexandre Richard*[a]

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Table of contents

Abbreviations

The following abbreviations are used throughout the text of the SI file: AcOH, acetic acid; BBI, broadband inverse; BSA, bovine serum albumin; DHX, dihydroxanthene; DIEA, *N*,*N* diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; ESI, electrospray ionization; FA, formic acid; HRMS, high-resolution mass spectrum; HATU, *N*-[(dimethylamino)-1H-1,2,3-triazolo-[4,5-b]pyridin-1-ylmethylene]-*N* methyl-methanaminium hexafluorophosphate *N*-oxide; HOAt, 1-hydroxy-7-azabenzotriazole; LRMS, low-resolution mass spectrum; MALDI-TOF, matrix-assisted laser desorption ionization - time-of-flight; NHS, *N*-hydroxysuccinimide; NIR, near-infrared; NMP, *N*-methyl-2-pyrrolidone; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; RP-HPLC, reversed-phase high-pressure liquid chromatography; TSTU, *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate; TEA; triethylamine; TEAB, triethylammonium bicarbonate; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate.

General

All reactions were carried out under a nitrogen or argon atmosphere with dry solvents under anhydrous conditions, unless otherwise noted. Reagents were purchased at the highest commercial quality and used without further purification. Yields refer to chromatographically and spectroscopically (^{1}H NMR) homogeneous materials. Reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25 mm E. Merck silica gel plates (60F-254) using UV light as visualizing agent and a solution of $KMnO_4$ and heat as developing agents. E. Merck silica gel (60, particle size 0.040-0.063 mm) was used for flash-column chromatography. Bovine serum albumin (BSA, heat shock fraction, pH 7, ≥ 98%) was provided by Sigma-Aldrich. Methanol (CH_3OH) was purchased in anhydrous form and used without further purification. DMF was dried over alumina cartridge using a solvent purification system. Water, ethyl acetate (EtOAc), diethyl ether (Et_2O), methylene chloride (CH_2Cl_2), and hexanes were purchased at the highest commercial quality and used without further purification. The HPLC-gradient grade methanol (CH_3OH) and acetonitrile (CH_3CN) were obtained from Carlo Erba and Biosolve respectively. Phosphate buffered saline (PBS, 100 mM phosphate + 150 mM NaCl, pH 7.4) and aq. mobile-phases for HPLC were prepared using water purified with a PURELAB Ultra system from ELGA (purified to 18.2 MΩ.cm). TEAB (1.0 M) buffer was prepared from distilled TEA and CO_2 gas. 2,3,3-trimethyl-3H-indole-5-carboxylic acid was prepared according to a literature procedure. [1] Diisopropylethylammonium salt of 2-aminoethane-1,1-disulfonic acid (this product being commercially available from Strem Chemicals Inc., Newburyport, MA, USA) was prepared using a protocol previously reported by us.[2]

Instruments and methods

^{1}H-, ^{13}C- and ^{19}F-NMR spectra of compounds **13**, **15**, **16** and **18** were recorded either on a

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[1] Pham, W.; Lai, W.-F.; Weissleder, R.; Tung, C.-H. *Bioconjugate Chem.* **2003**, *14*, 1048−1051.
[2] Romieu, A.; Tavernier-Lohr, D.; Pellet-Rostaing, S.; Lemaire, M.; Renard, P.-Y. *Tetrahedron Lett.* **2010**, *51*, 3304-3308.

Bruker Avance III 300 or 500 or on a Bruker Avance II 600 spectrometer (Dijon, France). All the other NMR spectra were recorded either on a Bruker AV-600 (600 MHz) or on a Bruker DRX-400 (400 MHz) instrument (Singapore). Chemical shifts are expressed in parts per million (ppm) from the residual non-deuterated solvent signal.[3] *J* values are expressed in Hz and the following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, m = multiplet, hex = hexet, br = broad. Infrared (IR) spectra were recorded with a Perkin-Elmer Spectrum One FTIR spectrometer with diamond ATR accessory or a universal ATR sampling accessory on a Bruker Alpha FT-IR spectrometer. The bond vibration frequencies are expressed in reciprocal centimeters (cm^{-1}). Elemental analyses (C, H, N, S) were perfomed on a Thermo Scientific Flash EA 1112 instrument. HPLC-MS analyses were performed on a Thermo-Dionex Ultimate 3000 instrument (pump + autosampler) equipped with a diode array detector (Thermo-Dionex DAD 3000-RS) and a MSQ Plus single quadrupole mass spectrometer (low-resolution mass (LRMS) analyses through electrospray ionization (ESI) source). Purifications by semi-preparative HPLC were performed on a Thermo-Dionex Ultimate 3000 instrument equipped with a RS Variable Detector (four distinct wavelengths). Some LRMS analyses (compounds **S7**, **S8** and crude NHS esters of **12**, **15** and **18**) were performed on a Bruker Amazon LS (ion trap) apparatus equipped with ESI source. High-resolution mass spectra (HRMS) were recorded on an Agilent ESI TOF (time-of-flight) mass spectrometer at 3.5 kV emitter voltage or a Thermo LTQ Orbitrap XL apparatus equipped with an electrospray ionisation (ESI) source. The purified BSA-DHX conjugates were characterized by MALDI-TOF mass spectrometry using a Bruker Ultraflex II LRF 2000 mass spectrometer (linear detector mode, sinapinic acid as a matrix, positive mode). Centrifugation operations were performed using a centrifuge UNIVERSAL 320 R from Hettich Lab Technology. UV-visible spectra were obtained either on a Varian Cary 50 scan (single-beam) or a JASCO V-530 (double beam) spectrophotometer by using a rectangular quartz cell (Hellma, 100-QS, 45 × 12.5 × 12.5 mm, pathlength 10 mm, chamber volume: 3.5 mL), at 25 °C (using a temperature control system combined with water circulation). Fluorescence spectra (emission/excitation spectra) were recorded with an HORIBA Jobin Yvon Fluorolog spectrophotometer (software FluorEssence) at 25 °C (using a temperature control system combined with water circulation), using a standard fluorometer cell (Labbox, LB Q, 10 mm). Emission spectra were recorded in the range 670–900 nm after excitation at 650 nm (shutter: Auto Open, excitation slit = 5 nm and emission slit = 5 nm). Excitation spectra were recorded in the range 400–810 nm (or 400–800 nm) after emission at 830 nm (or 820 nm) (shutter: Auto Open, excitation slit = 5 nm and emission slit = 12 nm). All fluorescence spectra were corrected until 850 nm. Fluorescence quantum yields were measured at 25 °C by a relative method using indocyanine green (ICG, Φ_F = 10.6% in DMSO) as a standard[4] (dilution by a factor of 3 between absorption and fluorescence measurements). The following equation was used to determine the relative fluorescence quantum yield:

$$\Phi_F(x) = (A_S/A_X)(F_X/F_S)(n_X/n_S)^2\Phi_F(s)$$

where A is the absorbance (in the range of 0.01–0.1 A.U.), F is the area under the emission curve, n is the refractive index of the solvents (at 25 °C) used in measurements, and the subscripts s and x represent standard and unknown, respectively. The following refractive index values were used: 1.479 for DMSO, 1.337 for PBS and PBS + 5% BSA. Stock

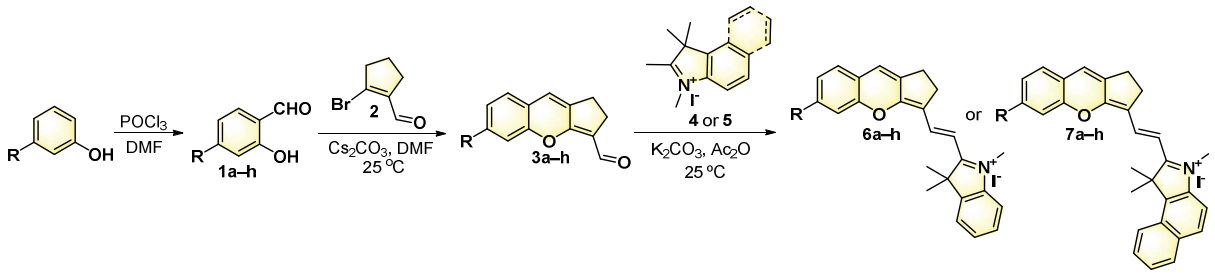
[3] Fulmer, G. R.; Miller, A. J. M.; Sherden, N. H.; Gottlieb, H. E.; Nudelman, A.; Stoltz, B. M.; Bercaw, J. E.; Goldberg, K. I. *Organometallics* **2010**, *29*, 2176–2179.
[4] Brouwer, A. M. *Pure Appl. Chem.* **2011**, *83*, 2213–2228.

solutions (1.0 mg/mL) of *nor*-DHX-hemicyanine fused NIR dyes were prepared in DMSO (+99.9%, for spectroscopy, Acros) and subsequently diluted with PBS or PBS + 5% BSA for UV-vis absorption and fluorescence measurements.

High-performance liquid chromatography (HPLC) separations

Four chromatographic systems were used for the analytical experiments and the purification steps respectively: *System A*: RP-HPLC (Phenomenex Kinetex C_{18} column, 2.6 µm, 2.1 × 50 mm) with CH_3CN (+ 0.1% FA) and 0.1% aq. formic acid (aq. FA, pH 2.7) as eluents [5% CH_3CN (0.1 min) followed by linear gradient from 5% to 100% (5 min) of CH_3CN] at a flow rate of 0.5 mL/min. UV-visible detection was achieved at 220, 260, 650 and 750 nm (+ diode array detection in the range 220-800 nm). Low resolution ESI-MS detection in the positive/negative mode (full scan, 150–1500 a.m.u., data type: centroid, needle voltage: 3.0 kV, probe temperature: 350 °C, cone voltage: 75 V and scan time: 1 s). *System B*: semi-preparative RP-HPLC (SiliCycle SiliaChrom C_{18} column, 10 µm, 20 × 250 mm) with CH_3CN and aq. 0.1% TFA (pH 2.0) as eluents [0% CH_3CN (5 min), followed by a gradient of 0% to 20% CH_3CN (10 min), then 20% to 80% CH_3CN (60 min)] at a flow rate of 20.0 mL/min. Quadruple UV-vis detection was achieved at 220, 260, 650 and 720 nm. *System C*: system B with aq. TEAB (50 mM, pH 7.5) as aq. mobile phase. *System D*: system C with the following gradient [0% CH_3CN (15 min), then 0% to 60% CH_3CN (60 min)] and quadruple UV detection at 240, 260, 280 and 300 nm.

Synthesized compounds



Scheme S1. General synthetic route towards *nor*-DHX-based fluorophores.

1) Preparation of salicylic aldehydes 1a–h.

1b and **1g** are commercially available and were purchased from Sigma-Aldrich and Tokyo Chemical Industry (TCI), respectively. **1a**,[5] **1c**,[6] **1d**[7] and **1h**[8] were prepared through the Vilsmeier-Haack reaction of the corresponding precursors.

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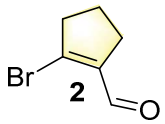
[5] Takakura, H.; Sasakura, K.; Ueno, T.; Urano, Y.; Terai, T.; Hanaoka, K.; Tsuboi, T.; Nagano, T. *Chem. Asian J.* **2010,** *5*, 2053–2061.

[6] Gurrapu, S.; Jonnalagadda, S. K.; Alam, M. A.; Ronayne, C. T.; Nelson, G. L.; Solano, L. N.; Lueth, E. A.; Drewes, L. R.; Mereddy, V. R. *Bioorg. Med. Chem.Lett.* **2016,** *26*, 3282–3286.

[7] Grimm, J. B.; English, B. P.; Chen, J.; Slaughter, J. P.; Zhang, Z.; Revyakin, A.; Patel, R.; Macklin, J. J.; Normanno, D.; Singer, R. H.; Lionnet, T.; Lavis, L. D. *Nat. Methods* **2015,** *12*, 244–250.

[8] Nizamov, S.; Willig, K. I.; Sednev, M. V.; Belov, V. N.; Hell, S. W. *Chem. Eur. J.* **2012,** *18*, 16339–16348.

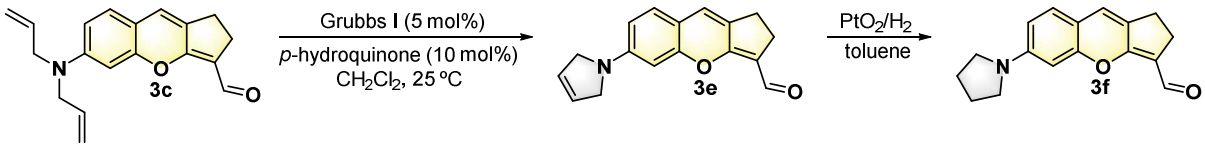


2-Bromocyclopent-1-ene-1-carbaldehyde (2): To DMF (26 mL, 339 mmol) and $CHCl_3$ (100 mL) at 0 °C was slowly added PBr_3 (26.8 mL, 282.5 mmol). After 60 min, cyclopentanone (10 mL, 113 mmol) in solution in $CHCl_3$ (15 mL) was added and the mixture was stirred for 16 h at 25 °C. The resulting red solution was then poured onto ice and solid $NaHCO_3$ was slowly added until pH ~ 7. The layers were separated and the aqueous layer was extracted with CH_2Cl_2 (100 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The chemical purity of the crude yellow oil was good and could be used directly in the next step. Aldehyde **2** was found to be rather volatile and unstable for prolonged storage at 25 °C and −20 °C and was thus stored under N_2 below −78 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ = 9.90 (s, 1H), 2.90 (s, 2H), 2.53 (s, 2H), 2.13–1.88 (m, 2H); ^{13}C NMR (151 MHz, $CDCl_3$) δ = 189.6, 141.8, 140.4, 42.9, 29.6, 21.7 ppm. The spectral data matched that previously obtained.[9]

2) Procedure for the synthesis of *nor*-DHX aldehydes 3a–h.

To a solution of salicylic aldehydes **1a–d**, **1g** or **1h** in dry DMF at 25 °C were added Cs_2CO_3 (3 eq.) and crude 2-bromocyclopent-1-ene-1-carbaldehyde **2** (2 eq.) in solution in dry DMF. The resulting reaction mixture was stirred for 48 h at 25 °C to reveal an intense yellow spot (TLC hexane/EtOAc, 8:2, v/v). The insoluble were then filtered on a pad of silica gel and the filtrate was concentrated under vacuum. The resulting residue was dissolved in CH_2Cl_2 and washed with deionized water. The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated in vacuo. Purification by flash-column chromatography on silica gel (CH_2Cl_2/EtOAc, 9:1, v/v) provided the desired aldehydes **3a–d**, **3g** or **3h** as deep orange solids.

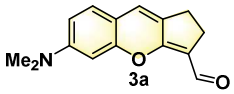
Salicylic aldehydes **3e** and **3f** were prepared according to the following sequence:



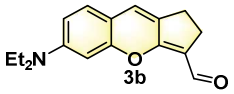
Scheme S2. Synthesis of *nor*-DHX aldehydes **3e** and **3f** from **3c**.

To a solution of aldehyde **3c** (37 mg, 0.13 mmol) in CH_2Cl_2 (4 mL) were added 1st generation Grubbs' catalyst (5.2 mg, 0.0063 mmol, 0.05 eq.) and *p*-benzoquinone (1.4 mg, 0.013 mmol, 0.1 eq.). The reaction mixture was stirred at 25 °C for 1 h before it was filtered over a short pad of silica gel. The filtrate was concentrated under vacuum and provided **3e** which was used directly in the next step, either for the formation of dyes **6e** and **7e** (*vide infra*) or hydrogenated in toluene for 1 h in the presence of Adam's catalyst ($PtO_2 \bullet H_2O$). After removing the catalyst on a short pad of silica gel, the filtrate was concentrated and cleanly afforded aldehyde **3f** which was used without further purification in the formation of *nor*-DHX dyes **6f** and **7f**. For analytical purposes, **3e** and **3f** were purified by flash-column chromatography on silica gel (CH_2Cl_2/EtOAc, 9:1, v/v) to provide analytically pure samples.

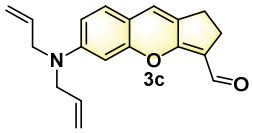
[9] Andrä, M. S.; Tzschucke, C. C. *Eur. J. Org. Chem.* **2014**, 7265–7272.



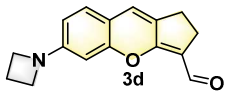
3a: 47% yield; dark orange solid; R_f = 0.5 (hexanes/EtOAc, 8:2, v/v); IR (film) ν_{max} = 2924, 1637, 1597, 1562, 1542, 1435, 1414, 1381, 1366, 1339, 1293, 1236, 1185, 1129, 1071, 867, 811 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$): δ = 9.99 (s, 1H), 7.07–6.90 (m, 1H), 6.57 (s, 1H), 6.48–6.45 (m, 2H), 3.01 (s, 6H), 2.76–2.66 (m, 4H); ^{13}C NMR (151 MHz, $CDCl_3$): δ = 184.2, 165.4, 153.6, 151.8, 134.9, 127.7, 123.6, 115.8, 111.8, 108.9, 99.3, 40.7 (2C), 24.6, 23.9 ppm; HRMS (ESI+): *m/z* calcd for $C_{15}H_{16}NO_2^+$ $[M + H]^+$ 242.1181, found 242.1182.



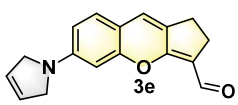
3b: 67% yield; dark orange solid; R_f = 0.5 (hexanes/EtOAc, 8:2, v/v); IR (film) ν_{max} = 2975, 1638, 1577, 1523, 1376, 1340, 1302, 1263, 1237, 1203, 1181, 1123, 825, 795, 771 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$): δ = 9.98 (s, 1H), 6.97 (d, *J* = 9.3 Hz, 1H), 6.56 (s, 1H), 6.48–6.31 (m, 2H), 3.38 (q, *J* = 7.1 Hz, 4H), 2.70 (s, 4H), 1.19 (t, *J* = 7.1 Hz, 6H); ^{13}C NMR (151 MHz, $CDCl_3$): δ = 184.1, 165.6, 154.0, 149.4, 134.1, 127.9, 123.7, 115.5, 111.1, 108.4, 98.5, 44.9 (2C), 24.6, 23.8, 12.9 (2C) ppm; HRMS (ESI+): *m/z* calcd for $C_{17}H_{20}NO_2^+$ $[M + H]^+$ 270.1494, found 270.1486.



3c: 72% yield; dark orange solid; R_f = 0.5 (hexanes/EtOAc, 8:2, v/v); IR (film) ν_{max} = 2321, 1674, 1633, 1604, 1571, 1521, 1435, 1417, 1376, 1338, 1236, 1193, 1177, 1126, 992, 959, 921 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$): δ = 9.96 (s, 1H), 6.95 (d, *J* = 9.2 Hz, 1H), 6.54 (s, 1H), 6.44 (dd, *J* = 4.6, 2.2 Hz, 2H), 5.84 (dd, *J* = 17.2, 10.4 Hz, 2H), 5.24–5.10 (m, 4H), 3.94 (d, *J* = 4.6 Hz, 4H), 2.68 (s, 4H); ^{13}C NMR (151 MHz, $CDCl_3$): δ = 184.2, 165.3, 153.6, 150.3, 134.8, 133.1 (2C), 127.7, 123.4, 116.8 (2C), 115.7, 112.0, 109.0, 99.4, 53.1 (2C), 24.6, 23.8 ppm; HRMS (ESI+): *m/z* calcd for $C_{19}H_{20}NO_2^+$ $[M + H]^+$ 294.1494, found 293.1496.

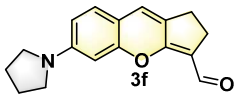


3d: 48% yield; dark orange solid; R_f = 0.5 (hexanes/EtOAc, 8:2, v/v); IR (film) ν_{max} = 2923, 2857, 1634, 1573, 1481, 1371, 1337, 1307, 1287, 1264, 1236, 1196, 1173, 1133, 826 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$): δ = 9.96 (s, 1H), 6.97 (d, *J* = 8.1 Hz, 1H), 6.57 (s, 1H), 6.21–6.13 (m, 2H), 3.94 (t, *J* = 7.3 Hz, 4H), 2.76–2.66 (m, 4H), 2.45–2.36 (m, 2H); ^{13}C NMR (151 MHz, $CDCl_3$): δ = 184.2, 165.4, 153.5, 153.1, 134.9, 127.8, 123.8, 115.7, 112.5, 107.9, 98.2, 52.3 (2C), 24.6, 23.8, 16.9 ppm; HRMS (ESI+): *m/z* calcd for $C_{16}H_{16}NO_2^+$ $[M + H]^+$ 254.1181, found 254.1184.

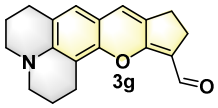


3e: —; dark orange solid; R_f = 0.5 (hexanes/EtOAc, 8:2, v/v); IR (film) ν_{max} = 2927, 2857, 1632, 1559, 1456, 1435, 1364, 1321, 1281, 1261, 1217, 1167 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$): δ = 9.97 (s, 1H), 7.02 (d, *J* = 9.1 Hz, 1H), 6.60 (s, 1H), 6.30 (s, 2H), 5.97 (s, 2H),

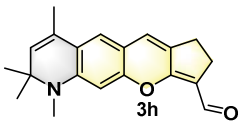
4.14 (s, 4H), 2.71 (s, 4H); ^{13}C NMR (151 MHz, $CDCl_3$): δ = 184.0, 165.7, 153.8, 148.5, 134.3, 128.1, 126.4 (2C), 124.1, 115.6, 111.5, 108.5, 98.5, 54.9 (2C), 24.6, 23.8 ppm; HRMS (ESI+): *m/z* calcd for $C_{17}H_{16}NO_2^+$ [M + H]$^+$ 266.1181, found 266.1177.



3f: —; dark orange solid; R_f = 0.5 (hexanes/EtOAc, 8:2, v/v); IR (film) ν_{max} = 2929, 2853, 1637, 1572, 1486, 1455, 1434, 1418, 1372, 1335, 1287, 1268, 1234, 1182, 1132 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 9.96 (s, 1H), 6.98 (d, *J* = 9.0 Hz, 1H), 6.58 (s, 1H), 6.32 (s, 2H), 3.32 (s, 4H), 2.70 (s, 4H), 2.03 (s, 4H); ^{13}C NMR (151 MHz, $CDCl_3$): δ = 183.8, 165.4, 153.4, 149.0, 133.6, 127.5, 123.8, 115.2, 110.9, 108.5, 98.4, 47.8 (2C), 25.5 (2C), 24.3, 23.5 ppm; HRMS (ESI+): *m/z* calcd for $C_{17}H_{18}NO_2^+$ [M + H]$^+$ 268.1338, found 268.1333.



3g: 65% yield; dark orange solid; R_f = 0.5 (hexanes/EtOAc, 8:2, v/v); IR (film) ν_{max} = 3422, 2948, 1631, 1578, 1466, 1395, 1308, 1209, 1161, 770, 640 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 9.94 (s, 1H), 6.58 (s, 1H), 6.51 (s, 1H), 3.31–3.09 (m, 4H), 2.81 (t, *J* = 6.6 Hz, 2H), 2.75–2.66 (m, 6H), 2.02–1.90 (m, 4H); ^{13}C NMR (151 MHz, $CDCl_3$): δ = 183.5, 166.0, 148.8, 144.5, 133.4, 124.7, 124.4, 118.1, 115.1, 111.1, 108.0, 50.3, 49.8, 27.6, 24.6, 23.8, 22.0, 21.2, 20.7 ppm; HRMS (ESI+): *m/z* calcd for $C_{19}H_{20}NO_2^+$ [M + H]$^+$ 294.1494, found 294.1492.

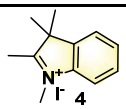


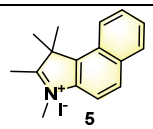
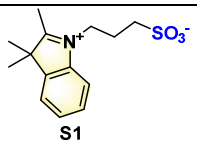
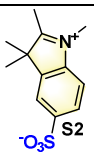
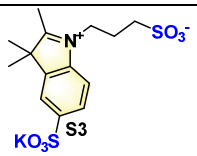
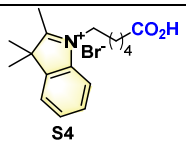
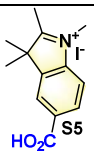
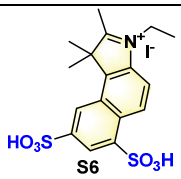
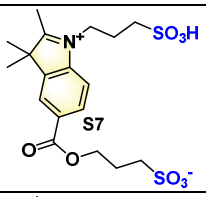
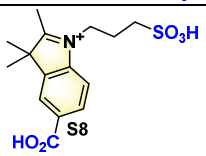
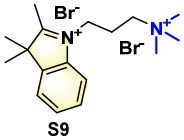
3h: 87% yield; dark orange solid; R_f = 0.5 (hexanes/EtOAc, 8:2, v/v); IR (film) ν_{max} = 2962, 2860, 1629, 1602, 1573, 1551, 1504, 1412, 1361, 1333, 1276, 1264, 1237, 1185, 1140 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 9.97 (s, 1H), 6.78 (s, 1H), 6.57 (s, 1H), 6.32 (s, 1H), 5.28 (d, *J* = 1.4 Hz, 1H), 2.85 (s, 3H), 2.71 (s, 4H), 1.97 (s, 3H), 1.34 (s, 6H); ^{13}C NMR (151 MHz, $CDCl_3$): δ = 183.9, 165.5, 153.6, 147.4, 134.5, 129.8, 127.1, 123.8, 121.4, 120.1, 115.4, 111.2, 98.0, 57.3, 31.5, 28.3, 24.6, 23.8, 18.9 (2C) ppm; HRMS (ESI+): *m/z* calcd for $C_{20}H_{22}NO_2^+$ [M + H]$^+$ 308.1651, found 308.1646.

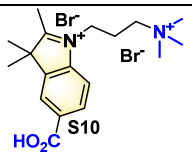
3) Synthesis of indolium and benzoindolium salts used in the synthesis of *nor*-DHX-hemicyanine fused dyes.

See Table S1 (*vide infra*) and the following protocol for the preparation of **S7–S8**.

Table S1. Indolium and benzoindolium salts used in the synthesis of *nor*-DHX-hemicyanine fused dyes

Chemical structure	CAS number	*nor*-DHX-based fluorophore(s) synthesized	Reference(s)
	5418-63-3	**6a–6h**	Richard, J.-A. *Org. Biomol. Chem.* **2015,** *13*, 8169–8172.

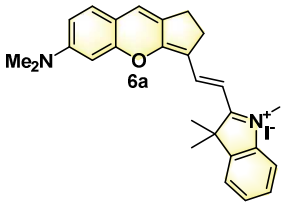
 5	58464-25-8	**7a–7h**	Richard, J.-A. *Org. Biomol. Chem.* **2015,** *13*, 8169–8172.
 S1	29636-96-2 (inner salt)	**8**	Mason, S. J.; Hake, J. L.; Nairne, J.; Cummins, W. J.; Balasubramanian, S. *J. Org. Chem.* **2005,** *70*, 2939–2949.
 S2	174703-04-9 (inner salt)	**9**	Mason, S. J.; Hake, J. L.; Nairne, J.; Cummins, W. J.; Balasubramanian, S. *J. Org. Chem.* **2005,** *70*, 2939–2949.
 S3	427882-78-8 (K^+ salt)	**10**	Richard, J.-A.; Massonneau, M.; Renard, P.-Y.; Romieu, A. *Org. Lett.* **2008,** *10*, 4175–4178.
 S4	171429-43-9 (Br^- salt)	**11**	Tan, X.; Luo, S.; Wang, D.; Su, Y.; Cheng, T.; Shi, C. *Biomaterials* **2012,** *33*, 2230–2239.
 S5	52302-32-6 (I^- salt)	**12**	(a) Wan, S.; Zheng, Y.; Shen, J.; Yang, W.; Yin, M. *ACS Appl. Mater. Interfaces* **2014**, *6*, 19515−19519; (b) Zhang, P.; Meng, J.; Li, X.; Matsuura, T.; Wang, Y. *J. Heterocycl. Chem.* **2002**, *39*, 179−184.
 S6	1628336-88-8 (I^- salt)	**13**	Mujumdar, S. R.; Mujumdar, R. B.; Grant, C. M.; Waggoner, A. S. *Bioconjugate Chem.* **1996,** *7*, 356–362.
 S7	-	**16**	Yue, S.; Shen, G.; Sun, D. *Preparation of indolylcyanine derivatives for use as dyes* (Pacific Biosciences of California, Inc.), WO 2012027623
 S8	212792-85-3 (inner salt)	**15**	Yue, S.; Shen, G.; Sun, D. *Preparation of indolylcyanine derivatives for use as dyes* (Pacific Biosciences of California, Inc.), WO 2012027623
 S9	1315275-89-8 (2× Br^- salt)	**17**	a) Choi, H. S.; Nasr, K.; Alyabyev, S.; Feith, D.; Lee, J. H.; Kim, S. H.; Ashitate, Y.; Hyun, H.; Patonay, G.; Strekowski, L.; Henary, M.; Frangioni, J. V. *Angew. Chem. Int. Ed.* **2011,** *50*, 6258–6263; b) Nanjunda,

			R.; Owens, E. A.; Mickelson, L.; Dost, T. L.; Stroeva, E. M.; Huynh, H. T.; Germann, M. W.; Henary, M. M.; Wilson, W. D. *Molecules* **2013**, *18*, 13588–13607.
	1420217-43-1 (2× Br^- salt)	**18**	Nanjunda, R.; Owens, E. A.; Mickelson, L.; Dost, T. L.; Stroeva, E. M.; Huynh, H. T.; Germann, M. W.; Henary, M. M.; Wilson, W. D. *Molecules* **2013**, *18*, 13588–13607.

A mixture of 2,3,3-trimethyl-3*H*-indole-5-carboxylic acid (300 mg, 1.5 mmol) and 1,3-propanesultone (1.55 mL, 17.7 mmol, 12 eq.) was heated in a sealed tube at 145 °C (oil bath temperature) for 12 h. After cooling at 25 °C, the reaction mixture was triturated in EtOAc (3 × 50 mL) to remove excess of 1,3-propanesultone. The resulting gummy solid was dissolved in CH_3OH, transferred into a 100-mL round-bottom flask and evaporated to dryness to give **S7**. LRMS (ESI+): *m/z* 448.0 $[M + H]^+$, calcd for $C_{18}H_{26}NO_8S_2^+$ 448.1; LRMS (ESI-): *m/z* 446.0 $[M - H]^-$, calcd for $C_{18}H_{24}NO_8S_2^-$ 446.1. To obtain carboxylic acid derivative **S8**, crude **S7** was dissolved in a (1:1, v/v) mixture of deionized water and aq. 37% HCl (20 mL) and the resulting reaction mixture was heated at 50–60 °C for 4 h. Thereafter, the mixture was partially evaporated, neutralized with aq. 40% NaOH to reach ca. pH 8, diluted with 1.0 M TEAB and purified by semi-preparative RP-HPLC (system D, 2 injections, t_R = 27.0-32.0 min). The product-containing fractions were lyophilized three times to give the TEA salt of compound **S8** (yield 74%). LRMS (ESI+): *m/z* 427.1 $[M + H + TEA]^+$, calcd for $C_{15}H_{20}NO_5S^+$ 326.1; LRMS (ESI-): *m/z* 324.0 [M - H]-, calcd for $C_{15}H_{18}NO_5S^-$ $[M - H]^-$ 324.1.

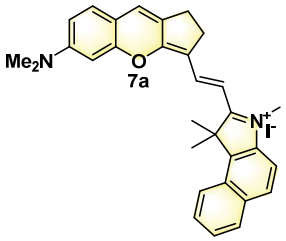
4) General procedure for the synthesis of *nor*-DHX-hemicyanine fused dyes 6a–h and 7a–h.

To *nor*-DHX aldehydes **3a–h** in anhydrous Ac_2O (0.025–0.05 M) were added 1,2,3,3-tetramethyl-3*H*-indolium iodide **4** or 1,1,2,3-tetramethyl-1*H*-benz[*e*]indolium iodide **5** (1.2 eq.) and K_2CO_3 (2 eq.) and the mixture was stirred at 25 °C for 16 h to reveal an intense green spot (TLC CH_2Cl_2/CH_3OH, 9:1, v/v). The reaction mixture was concentrated and the resulting residue dissolved in CH_2Cl_2 and washed with deionized water. The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. Purification by flash-column chromatography on silica gel (step gradient of CH_3OH in CH_2Cl_2 from 0% to 3%) afforded NIR *nor*-DHX-based fluorophores **6a–h** and **7a–h**.

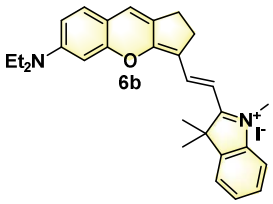


6a: 95% yield; dark green solid; R_f = 0.5 (CH_2Cl_2/CH_3OH, 9:1, v/v); IR (film) ν_{max} = 3423, 2928, 1717, 1631, 1541, 1515, 1490, 1458, 1444, 1400, 1381, 1354, 1303, 1285, 1259, 1218, 1173, 1157, 1110, 1043, 1017, 928, 808, 752 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$): δ = 8.11 (d, *J* = 14.1 Hz, 1H), 7.46–7.34 (m, 4H), 7.27–7.19 (m, 3H), 6.76–6.71 (m, 1H), 6.03 (d, *J* = 14.1

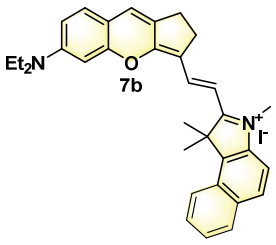
Hz, 1H), 3.80 (s, 3H), 3.17 (s, 6H), 3.01 (s, 4H), 1.75 (s, 6H); ^{13}C NMR (151 MHz, $CDCl_3$): δ = 173.9, 168.5, 155.6, 153.5, 142.7, 141.0, 138.9, 133.6, 133.3, 129.4, 128.9, 125.8, 122.4, 121.7, 113.7, 112.0, 111.3, 101.8, 98.1, 49.7, 40.7 (2C), 33.0, 28.4 (2C), 25.6, 25.1 ppm; HRMS (ESI+): *m/z* calcd for $C_{27}H_{29}N_2O^+$ $[M]^{+\circ}$ 397.2274, found 397.2256.



7a: 92% yield; dark green solid; R_f = 0.5 (CH_2Cl_2/CH_3OH, 9:1, v/v); IR (film) ν_{max} = 3410, 2927, 1631, 1603, 1543, 1512, 1476, 1444, 1401, 1352, 1291, 1245, 1207, 1172, 1127, 1073, 1016, 940, 898, 815, 767, 751 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.23 (d, *J* = 14.3 Hz, 1H), 8.15 (d, *J* = 8.6 Hz, 1H), 7.99–7.90 (m, 2H), 7.64–7.59 (m, 1H), 7.55–7.44 (m, 2H), 7.37–7.20 (s, 2H), 6.78 (s, 1H), 6.72 (d, *J* = 8.6 Hz, 1H), 6.08 (d, *J* = 14.3 Hz, 1H), 3.96 (s, 3H), 3.17 (s, 6H), 3.02 (d, *J* = 11.4 Hz, 4H), 2.03 (s, 6H); ^{13}C NMR (151 MHz, $CDCl_3$) δ = 175.9, 168.3, 155.7, 153.6, 140.3, 138.9, 134.5, 134.1, 132.5 (2C), 131.2, 130.5, 129.4, 128.3, 128.2, 125.7, 122.5, 121.7, 113.8, 112.0, 111.3, 102.2, 98.6, 51.8, 41.0 (2C), 34.3, 28.2 (2C), 26.1, 25.3 ppm; HRMS (ESI+): *m/z* calcd for $C_{31}H_{31}N_2O^+$ $[M]^{+\circ}$ 447.2431, found 447.2435.

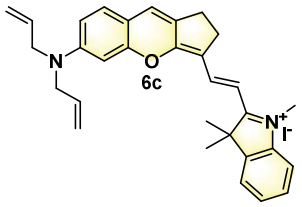


6b: 85% yield; dark green solid; R_f = 0.4 (CH_2Cl_2/CH_3OH, 9:1, v/v); IR (film) ν_{max} = 3444, 2974, 2383, 1631, 1542, 1512, 1458, 1391, 1308, 1256, 1217, 1168, 1114, 1075, 1045, 1016, 928, 808, 765, 750 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.10 (d, *J* = 14.1 Hz, 1H), 7.55–7.31 (m, 4H), 7.30–7.10 (m, 2H), 6.88–6.55 (m, 2H), 5.99 (d, *J* = 14.1 Hz, 1H), 3.78 (s, 3H), 3.52 (q, *J* = 7.1 Hz, 4H), 3.02 (s, 4H), 1.77 (s, 6H), 1.28 (t, *J* = 7.1 Hz, 6H); ^{13}C NMR (151 MHz, $CDCl_3$): δ = 173.6, 168.8, 156.3, 151.9, 143.0, 141.1, 138.7, 133.9, 133.4, 130.0, 129.1, 125.8, 122.6, 122.2, 113.8, 112.3, 111.3, 101.7, 97.7, 49.8, 45.5 (2C), 33.2, 28.6 (2C), 26.0, 25.3, 13.0 (2C) ppm; HRMS (ESI): *m/z* calcd for $C_{29}H_{33}N_2O^+$ $[M]^{+\circ}$ 425.2587, found 425.2584.

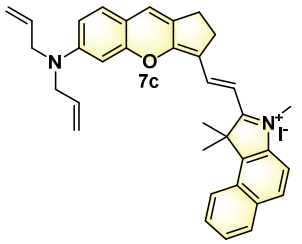


7b: 81% yield; dark green solid; R_f = 0.4 (CH_2Cl_2/CH_3OH, 9:1, v/v); IR (film) ν_{max} = 2983, 2383, 1631, 1542, 1510, 1443, 1390, 1368, 1351, 1275, 1259, 1240, 1207, 1167, 1128, 1075, 1015, 939, 765, 750 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.21 (d, *J* = 14.1 Hz, 1H), 8.15 (d, *J* = 8.4 Hz, 1H), 7.94 (dd, *J* = 11.9, 8.5 Hz, 2H), 7.61 (ddd, *J* = 8.3, 6.7, 1.3 Hz, 1H), 7.53 (d, *J* = 8.7 Hz, 1H), 7.47 (dd, *J* = 8.2, 6.7 Hz, 1H), 7.33 (d, *J* = 9.1 Hz, 2H), 6.82–6.61 (m, 2H), 6.05 (d, *J* = 14.1 Hz, 1H), 3.95 (s, 3H), 3.52 (q, *J* = 7.2 Hz, 4H), 3.11–2.94 (m, 4H), 2.04 (s, 6H), 1.28 (t, *J* = 7.2 Hz, 6H); ^{13}C NMR (151 MHz, $CDCl_3$): δ = 175.4, 168.3, 156.2, 151.7,

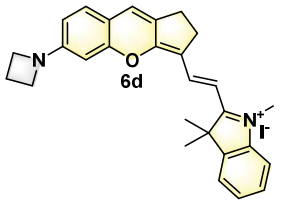
140.4, 138.3, 134.2, 133.5, 133.1, 132.4, 131.1, 130.5, 129.8, 128.3, 128.2, 125.6, 122.5, 121.8, 113.7, 112.0, 111.3, 101.7, 97.8, 51.6, 45.5 (2C), 34.0, 28.2 (2C), 26.0, 25.3, 13.0 (2C) ppm; HRMS (ESI+): *m/z* calcd for $C_{33}H_{35}N_2O^+$ $[M]^{+\circ}$ 475.2744, found 475.2740.



6c: 60% yield; dark green solid; R_f = 0.4 (CH_2Cl_2/CH_3OH, 9:1, v/v); IR (film) ν_{max} = 3430, 1629, 1551, 1531, 1512, 1491, 1459, 1443, 1400, 1379, 1362, 1305, 1289, 1264, 1210, 1166, 1109, 1043, 1017, 926, 808 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$): δ = 8.02 (d, *J* = 14.1 Hz, 1H), 7.40 (d, *J* = 7.3 Hz, 1H), 7.36–7.26 (m, 3H), 7.21–7.16 (m, 2H), 6.70–6.59 (m, 2H), 5.94 (d, *J* = 14.1 Hz, 1H), 5.87–5.77 (m, 2H), 5.20–5.05 (m, 4H), 4.00 (d, *J* = 4.7 Hz, 4H), 3.73 (s, 3H), 2.93 (s, 4H), 1.69 (s, 6H); ^{13}C NMR (151 MHz, $CDCl_3$): δ = 174.1, 168.3, 155.4, 152.3, 142.5, 141.0, 139.0, 133.8, 133.0, 131.9(1), 131.8(9), 129.5, 128.8, 125.9, 122.4, 121.5, 117.0 (2C), 113.9, 112.3, 111.3, 101.9, 98.2, 53.0, 49.7 (2C), 33.2, 28.3 (2C), 25.7, 25.0 ppm; HRMS (ESI+): *m/z* calcd for $C_{31}H_{33}N_2O^+$ $[M]^{+\circ}$ 449.2587, found 449.2585.

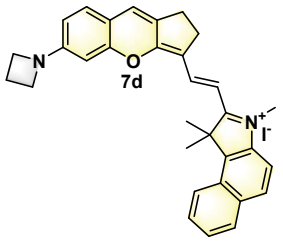


7c: 68% yield; dark green solid; R_f = 0.4 (CH_2Cl_2/CH_3OH, 9:1, v/v); IR (film) ν_{max} = 3410, 1629, 1548, 1532, 1509, 1474, 1443, 1401, 1386, 1362, 1295, 1255, 1229, 1203, 1166, 1127, 1016, 939, 898 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$): δ = 8.26 (d, *J* = 14.3 Hz, 1H), 8.20 (d, *J* = 8.6 Hz, 1H), 8.03–7.96 (m, 2H), 7.69–7.64 (m, 1H), 7.60 (d, *J* = 8.6 Hz, 1H), 7.56–7.50 (m, 1H), 7.37–7.27 (m, 2H), 6.77 (d, *J* = 3.7 Hz, 2H), 6.17 (d, *J* = 14.3 Hz, 1H), 6.00–5.90 (m, 2H), 5.32–5.21 (m, 4H), 4.12 (s, 4H), 4.03 (s, 3H), 3.13–3.01 (m, 4H), 2.07 (s, 6H); ^{13}C NMR (151 MHz, $CDCl_3$): δ = 176.1, 168.1, 155.6, 152.4, 140.2, 138.9, 134.5, 134.4, 132.5, 132.3 (2C), 132.1, 131.2, 130.5, 129.5, 128.2 (2C), 125.7, 122.5, 121.6, 117.3 (2C), 114.1, 112.2, 111.4, 102.4, 98.7, 53.3 (2C), 51.8 , 34.3, 28.1 (2C), 26.1, 25.2 ppm; HRMS (ESI+): *m/z* calcd for $C_{35}H_{35}N_2O^+$ $[M]^{+\circ}$ 499.2744, found 499.2747.

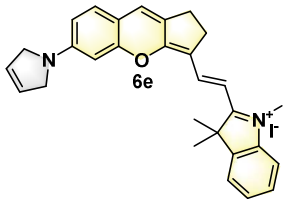


6d: 85% yield; dark green solid; R_f = 0.4 (CH_2Cl_2/CH_3OH, 9:1, v/v); IR (film) ν_{max} = 3423, 2931, 2868, 1630, 1542, 1514, 1457, 1401, 1378, 1357, 1285, 1257, 1215, 1157, 1109, 1044, 1016, 928, 804 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ = 8.11 (d, *J* = 14.1 Hz, 1H), 7.46–7.39 (m, 2H), 7.39–7.35 (m, 1H), 7.32 (d, *J* = 8.4 Hz, 1H), 7.28–7.20 (m, 2H), 6.44–6.35 (m, 2H), 6.06 (d, *J* = 14.1 Hz, 1H), 4.13 (t, *J* = 7.4 Hz, 4H), 3.80 (s, 3H), 3.02 (s, 4H), 2.55–2.45 (m, 2H), 1.76 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 174.2, 168.6, 155.8, 154.2, 143.0, 141.3, 139.2, 133.8 (2C), 129.9, 129.2, 126.0, 122.6, 122.0, 114.5, 111.5, 110.9, 102.1, 96.8, 52.1

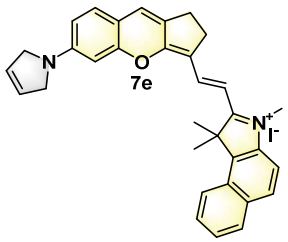
(2C), 49.9, 33.1, 28.7 (2C), 25.9, 25.4, 16.7 ppm; HRMS (ESI+): *m/z* calcd for $C_{28}H_{29}N_2O^+$ $[M]^{+\circ}$ 409.2274, found 409.2269.



7d: 78% yield; dark green solid; R_f = 0.4 (CH_2Cl_2/CH_3OH, 9:1, v/v); IR (film) ν_{max} = 3395, 1630, 1542, 1512, 1469, 1451, 1401, 1357, 1288, 1243, 1288, 1243, 1205, 1161, 1138, 1128, 1016, 939 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 8.24 (d, *J* = 14.3 Hz, 1H), 8.15 (d, *J* = 8.5 Hz, 1H), 7.96 (t, *J* = 7.6 Hz, 2H), 7.67–7.59 (m, 1H), 7.57–7.45 (m, 2H), 7.31–7.23 (m, 2H), 6.52 (s, 1H), 6.38 (dd, *J* = 8.5, 2.0 Hz, 1H), 6.15 (d, *J* = 14.3 Hz, 1H), 4.14 (t, *J* = 7.4 Hz, 4H), 4.01 (s, 3H), 3.11–2.97 (m, 4H), 2.56–2.44 (m, 2H), 2.04 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 175.7, 167.8, 155.3, 153.8, 140.0, 138.5, 134.1, 133.6, 132.3, 132.2, 130.9, 130.2, 129.2, 128.0, 127.9, 125.4, 122.2, 121.4, 114.0, 111.0, 110.3, 102.0, 96.9, 51.9 (2C), 51.5, 34.1, 27.9 (2C), 25.8, 25.0, 16.4 ppm; HRMS (ESI+): *m/z* calcd for $C_{32}H_{31}N_2O^+$ $[M]^{+\circ}$ 459.2431, found 459.2428.

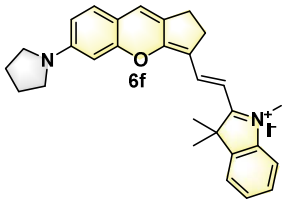


6e: 64% yield over 2 steps; dark green solid; R_f = 0.4 (CH_2Cl_2/CH_3OH, 9:1, v/v); IR (film) ν_{max} = 3431, 1636, 1547, 1537, 1513, 1446, 1400, 1354, 1284, 1256, 1213, 1155, 1106, 1043, 1016, 927, 809 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$): δ = 8.11 (d, *J* = 14.2 Hz, 1H), 7.45 (dd, *J* = 7.5, 1H), 7.43–7.35 (m, 3H), 7.31–7.18 (m, 2H), 6.64–6.57 (m, 2H), 6.03–5.98 (m, 3H), 4.27 (s, 4H), 3.79 (s, 3H), 3.01 (s, 4H), 1.76 (s, 6H); ^{13}C NMR (151 MHz, $CDCl_3$): δ = 174.0, 168.6, 155.8, 150.6, 142.9, 141.2, 139.0, 133.8, 133.5, 130.0, 129.1, 126.1 (2C), 126.0, 122.7, 121.9, 114.0, 112.4, 111.4, 101.9, 98.2, 55.4 (2C), 49.9, 33.1, 28.6 (2C), 25.8, 25.3 ppm; HRMS (ESI+): *m/z* calcd for $C_{29}H_{29}N_2O^+$ $[M]^{+\circ}$ 421.2274, found 421.2275.

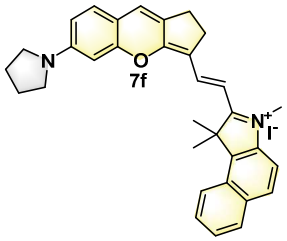


7e: 50% yield over two steps; dark green solid; R_f = 0.4 (CH_2Cl_2/CH_3OH, 9:1, v/v); IR (film) ν_{max} = 3442, 2928, 2852, 1637, 1542, 1512, 1446, 1402, 1352, 1289, 1242, 1205, 1161, 1138, 1126, 1016, 938, 898 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$): δ = δ 8.25 (d, *J* = 14.3 Hz, 1H), 8.16 (d, *J* = 8.5 Hz, 1H), 7.95 (t, *J* = 8.8 Hz, 2H), 7.66–7.59 (m, 1H), 7.56–7.45 (m, 2H), 7.36–7.19 (m, 2H), 6.70 (s, 1H), 6.58 (d, *J* = 8.5 Hz, 1H), 6.10 (d, *J* = 14.3 Hz, 1H), 6.02 (s, 2H), 4.29 (s, 4H), 3.98 (s, 3H), 3.08–2.98 (m, 4H), 2.04 (s, 6H); ^{13}C NMR (151 MHz, $CDCl_3$): δ = 175.8, 168.2, 155.7, 150.5, 140.3, 138.8, 134.3, 133.8, 132.8, 132.5, 131.2, 130.5, 129.7, 128.3, 128.2, 126.2 (2C), 125.7, 122.5, 121.7, 113.9, 112.0, 111.3, 102.1, 98.5, 55.4 (2C),

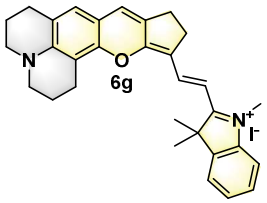
51.8, 34.2, 28.2 (2C), 26.0, 25.3 ppm; HRMS (ESI+): *m/z* calcd for $C_{33}H_{31}N_2O^+$ $[M]^{+\circ}$ 471.2431, found 471.2424.



6f: 84% yield over three steps; dark green solid; R_f = 0.4 (CH_2Cl_2/CH_3OH, 9:1, v/v); IR (film) ν_{max} = 3431, 2967, 2926, 2856, 1632, 1541, 1512, 1447, 1399, 1379, 1357, 1346, 1300, 1283, 1256, 1213, 1155, 1109, 1043, 1016, 927, 807 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$): δ = 8.11 (d, *J* = 14.1 Hz, 1H), 7.44–7.36 (m, 3H), 7.36 (d, *J* = 8.7 Hz, 1H), 7.24–7.21 (m, 1H), 7.19 (d, *J* = 7.9 Hz, 1H), 6.68–6.62 (m, 2H), 5.99 (d, *J* = 14.1 Hz, 1H), 3.78 (s, 3H), 3.49 (s, 4H), 3.03 (s, 4H), 2.11 (s, 4H), 1.76 (s, 6H); ^{13}C NMR (151 MHz, $CDCl_3$): δ = 173.1, 168.6, 155.7, 151.1, 142.7, 140.8, 138.3, 134.0, 132.9, 129.6, 128.8, 125.4, 122.3, 121.9, 113.8, 112.7, 110.9, 101.3, 98.0, 49.4, 48.4 (2 C), 29.7 28.4 (2C), 25.7, 25.5 (2C), 25.1 ppm; HRMS (ESI+): *m/z* calcd for $C_{29}H_{31}N_2O^+$ $[M]^{+\circ}$ 423.2431, found 423.2419.

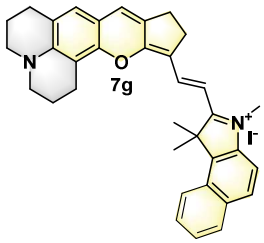


7f: 73% yield over three steps; dark green solid; R_f = 0.4 (CH_2Cl_2/CH_3OH, 9:1, v/v); IR (film) ν_{max} = 3431, 2934, 2856, 1632, 1541, 1509, 1447, 1401, 1358, 1288, 1242, 1205, 1158, 1125, 1016, 939, 898 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 8.24 (d, *J* = 14.3 Hz, 1H), 8.15 (d, *J* = 8.5 Hz, 1H), 8.00–7.91 (m, 2H), 7.67–7.58 (m, 1H), 7.54–7.45 (m, 2H), 7.37–7.29 (m, 2H), 6.72 (s, 1H), 6.65–6.58 (m, 1H), 6.08 (d, *J* = 14.3 Hz, 1H), 3.97 (s, 3H), 3.55–3.44 (m, 4H), 3.11–2.97 (m, 4H), 2.16–2.07 (m, 4H), 2.04 (s, 6H); ^{13}C NMR (151 MHz, $CDCl_3$): δ = 175.4, 168.5, 155.9, 151.4, 140.4, 138.4, 134.2, 133.4, 133.4, 132.5, 131.2, 130.5, 129.7, 128.4, 128.2, 125.6, 122.5, 121.9, 114.0, 112.7, 111.3, 101.7, 98.6, 51.7, 48.8 (2C), 34.1, 28.3 (2C), 26.1, 25.8 (2C), 25.4 ppm; HRMS (ESI+): *m/z* calcd for $C_{33}H_{33}N_2O^+$ $[M]^{+\circ}$ 473.2587, found 473.2582.

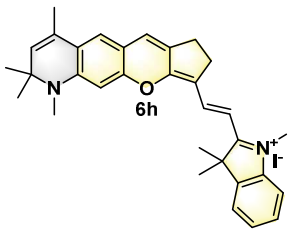


6g: 92% yield; dark green solid; R_f = 0.4 (CH_2Cl_2/CH_3OH, 9:1, v/v); IR (film) ν_{max} 3444, 1631, 1517, 1493, 1451, 1394, 1312, 1299, 1277, 1259, 1203, 1175, 1153, 1112, 1043, 1018, 928, 805, 765, 750 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$): δ = 7.93 (d, *J* = 13.9 Hz, 1H), 7.47 (s, 1H), 7.38–7.26 (m, 2H), 7.13 (t, *J* = 7.5 Hz, 1H), 7.06 (d, *J* = 7.9 Hz, 1H), 7.00 (s, 1H), 5.76 (d, *J* = 13.9 Hz, 1H), 3.59 (s, 3H), 3.40 (dt, *J* = 16.8, 5.8 Hz, 4H), 3.02–2.95 (m, 2H), 2.97–2.85 (m, 4H), 2.76 (t, *J* = 6.3 Hz, 2H), 2.12–1.99 (m, 2H), 1.99–1.88 (m, 2H), 1.67 (s, 6H); ^{13}C NMR (151 MHz, $CDCl_3$): δ = 171.1, 168.6, 151.4, 148.2, 143.1, 140.4, 135.8, 135.8, 131.3, 128.8, 126.4, 124.7, 123.2, 122.5, 122.2, 114.5, 110.2, 106.0, 99.3, 50.6, 50.1, 48.8,

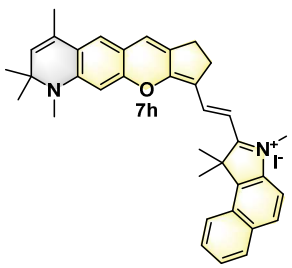
32.2, 28.7 (2C), 27.8, 25.4(5), 25.4(0), 21.0, 20.3, 19.9 ppm; HRMS (ESI+): *m/z* calcd for $C_{31}H_{33}N_2O^+$ $[M]^{+\circ}$ 449.2587, found 449.2596.



7g: 87% yield; dark green solid; R_f = 0.4 (CH_2Cl_2/CH_3OH, 9:1, v/v); IR (film) ν_{max} 3006, 2358, 1630, 1513, 1450, 1395, 1276, 1261, 1240, 1202, 1174, 1127, 1012, 939, 765, 750 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$): δ = 8.13 (d, *J* = 8.4 Hz, 1H), 8.08 (d, *J* = 14.0 Hz, 1H), 7.90 (dd, *J* = 8.5, 6.3 Hz, 2H), 7.58 (ddd, *J* = 8.4, 6.7, 1.3 Hz, 1H), 7.47–7.33 (m, 3H), 6.99 (s, 1H), 5.88 (d, *J* = 14.0 Hz, 1H), 3.78 (s, 3H), 3.43 (dt, *J* = 23.5, 5.7 Hz, 4H), 3.11–2.93 (m, 6H), 2.80 (t, *J* = 6.3 Hz, 2H), 2.16–2.10 (m, 2H), 2.00 (m, 8H); ^{13}C NMR (151 MHz, $CDCl_3$): δ = 173.3, 168.4, 151.4, 148.1, 140.6, 136.0, 135.0, 133.0, 132.0, 131.7, 131.0, 130.4, 128.4, 128.0, 126.4, 125.1, 122.9, 122.2(8), 122.3(2), 114.4, 110.9, 106.3, 99.6, 50.9, 50.7, 50.3, 32.9, 28.3 (2C), 27.9, 25.6, 25.5, 21.2, 20.5, 20.1 ppm; HRMS (ESI+): *m/z* calcd for $C_{35}H_{35}N_2O^+$ $[M]^{+\circ}$ 499.2744, found 499.2736.



6h: 75% yield; dark green solid; R_f = 0.4 (CH_2Cl_2/CH_3OH, 9:1, v/v); IR (film) ν_{max} = 3421, 2970, 2928, 1626, 1557, 1526, 1442, 1397, 1378, 1346, 1253, 1214, 1175, 1107, 1043, 1018, 927, 817, 796 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$): δ = 8.07 (d, *J* = 13.9 Hz, 1H), 7.46 (s, 1H), 7.41 (d, *J* = 7.3 Hz, 1H), 7.38–7.35 (m, 1H), 7.24–7.20 (m, 1H), 7.15 (d, *J* = 7.9 Hz, 1H), 7.08 (s, 1H), 6.67 (s, 1H), 5.91 (d, *J* = 13.9 Hz, 1H), 5.43 (s, 1H), 3.73 (s, 3H), 3.06 (s, 3H), 3.05–2.97 (m, 4H), 2.02 (s, 3H), 1.75 (s, 6H), 1.43 (s, 6H); ^{13}C NMR (151 MHz, $CDCl_3$): δ = 172.7, 168.4, 156.7, 150.4, 143.2, 141.0, 137.6, 134.3, 133.4, 132.0, 129.0, 126.5, 125.4, 122.9, 122.6, 122.4, 122.3, 114.6, 110.9, 100.9, 97.1, 58.8, 49.6, 33.0, 30.0, 29.4 (2C), 28.7 (2C), 25.9, 25.5, 19.1 ppm; HRMS (ESI+): *m/z* calcd for $C_{32}H_{35}N_2O^+$ $[M]^{+\circ}$ 463.2744, found 463.2745.

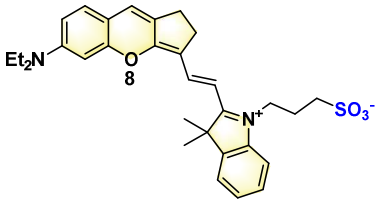


7h: 67% yield; dark green solid; R_f = 0.4 (CH_2Cl_2/CH_3OH, 9:1, v/v); IR (film) ν_{max} = 3412, 2970, 2928, 1703, 1625, 1604, 1556, 1532, 1518, 1474, 1443, 1398, 1347, 1278, 1259, 1239, 1205, 1174, 1126, 1015, 939 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$): δ = 8.20 (d, *J* = 14.1 Hz, 1H), 8.15 (d, *J* = 8.4 Hz, 1H), 7.97–7.90 (m, 2H), 7.64–7.57 (m, 1H), 7.51–7.43 (m, 2H), 7.37 (s, 1H), 7.04 (s, 1H), 6.73 (s, 1H), 6.00 (d, *J* = 14.1 Hz, 1H), 5.43 (s, 1H), 3.92 (s, 3H), 3.11–

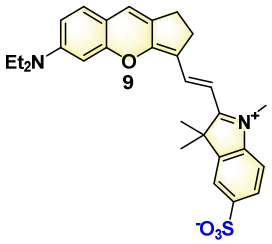
3.00 (m, 6H), 2.07–1.98 (m, 7H), 1.60 (s, 3H), 1.44 (s, 6H); ^{13}C NMR (151 MHz, $CDCl_3$) δ = 174.6, 168.1, 156.6, 150.2, 140.5, 137.5, 133.9, 133.6, 133.4, 132.3, 131.8, 131.0, 130.5, 128.5, 128.1, 126.5, 125.4, 122.8, 122.5, 122.1, 122.1, 114.4, 111.1, 101.0, 97.4, 58.8, 51.5, 33.0, 30.0, 29.4 (2C), 28.3 (2C), 26.0, 25.5, 19.1 ppm; HRMS (ESI+): *m/z* calcd for $C_{36}H_{37}N_4O_5^+$ $[M]^{+\circ}$ 513.2900, found 513.2902.

5) General procedure for the synthesis of water-soluble *nor*-DHX-hemicyanine fused dyes 8–12.

To *nor*-DHX aldehyde **3b** in anhydrous Ac_2O (0.025–0.1 M) were added the corresponding indolinium salt (1.2 eq.) and the mixture was stirred at 25 °C for 16 h to reveal an intense green spot (TLC CH_2Cl_2/CH_3OH, 9:1, v/v). The reaction mixture was concentrated and directly loaded on silica gel and purified (step gradient of CH_3OH in CH_2Cl_2 from 0% to 10–15%) to afford *nor*-DHX-based fluorophores **8–12**.

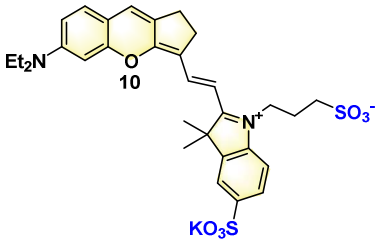


8: 88% yield; dark green solid; R_f = 0.5 (CH_2Cl_2/CH_3OH, 9:1, v/v); IR (film) ν_{max} = 3430, 2971, 2926, 1632, 1535, 1509, 1456, 1440, 1409, 1392, 1379, 1346, 1271, 1252, 1214, 1142, 1119, 1037, 1015, 929 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD): δ = 8.19 (d, *J* = 14.0 Hz, 1H), 7.57–7.44 (m, 2H), 7.40 (t, *J* = 4.1 Hz, 3H), 7.27 (t, *J* = 7.7 Hz, 1H), 6.86 (d, *J* = 8.0 Hz, 2H), 6.22 (d, *J* = 14.0 Hz, 1H), 4.43–4.26 (m, 2H), 3.54 (q, *J* = 6.9 Hz, 4H), 2.99 (s, 6H), 2.32–2.15 (m, 2H), 1.75 (s, 6H), 1.25 (t, *J* = 7.0 Hz, 6H); ^{13}C NMR (100 MHz, CD_3OD): δ = 174.5, 169.9, 157.4, 153.2, 143.4, 142.6, 139.8, 135.0, 134.5, 130.8, 129.9, 126.5, 123.4, 123.3, 115.0, 113.2, 112.3, 102.3, 98.3, 50.7, 46.0 (2C), 44.0, 30.7, 28.4 (2C), 26.2, 25.8, 24.0, 12.9 (2C) ppm; HPLC (system A): t_R = 4.9 min (purity 99% at 750 nm); LRMS (ESI+, recorded during RP-HPLC analysis): *m/z* 533.2 $[M + H]^+$ (100) and 1065.8 $[2M + H]^+$ (10), calcd for $C_{31}H_{37}N_2O_4S^+$ 533.2; HRMS (ESI+): *m/z* calcd for $C_{31}H_{37}N_2O_4S^+$ $[M + H]^+$ 533.2469, found 533.2483.

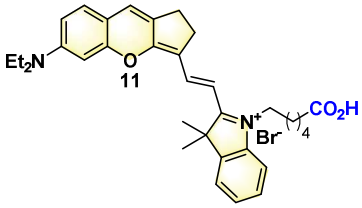


9: 59% yield; dark green solid; R_f = 0.5 (CH_2Cl_2/CH_3OH, 9:1, v/v); IR (film) ν_{max} = 3453, 2982, 2918, 2849, 2347, 1631, 1540, 1510, 1445, 1393, 1375, 1351, 1275, 1257, 1213, 1166, 1114, 1068, 1026, 931 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD): δ = 8.24 (d, *J* = 13.9 Hz, 1H), 7.89 (d, *J* = 10.0 Hz, 2H), 7.63 (s, 1H), 7.48 (d, *J* = 8.7 Hz, 1H), 7.31 (d, *J* = 8.1 Hz, 1H), 6.96 (m, 2H), 5.98 (d, *J* = 14.0 Hz, 1H), 3.68–3.52 (m, 7H), 3.01 (dd, *J* = 26.8, 7.9 Hz, 4H), 1.78 (s, 6H), 1.28 (t, *J* = 7.0 Hz, 6H); ^{13}C NMR (100 MHz, CD_3OD): δ = 174.6, 170.6, 157.8, 153.7, 145.7, 143.2, 142.1, 139.1, 136.6, 134.1, 131.1, 128.1, 123.7, 121.3, 115.5, 113.9, 111.2, 101.5, 98.3, 50.3, 46.1 (2C), 31.6, 28.3 (2C), 26.0, 25.8, 12.8 (2C) ppm; HPLC (system A): t_R = 4.6 min (purity 99% at 750 nm); LRMS (ESI+, recorded during RP-HPLC analysis): *m/z* 505.2 $[M + H]^+$ (100), calcd for $C_{29}H_{33}N_2O_4S^+$ 505.2; LRMS (ESI-, recorded during RP-

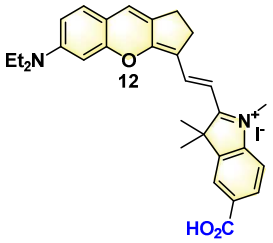
HPLC analysis): *m/z* 503.2 $[M - H]^-$ (100), calcd for $C_{29}H_{31}N_2O_4S^-$ 503.2; HRMS (ESI+): *m/z* calcd for $C_{29}H_{33}N_2O_4S^+$ $[M + H]^+$ 505.2156, found 505.2167.



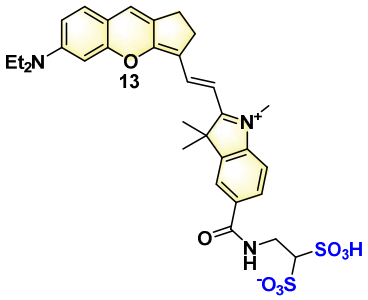
10: 53% yield; dark green solid; R_f = 0.2 (CH_2Cl_2/CH_3OH, 8:2, v/v); IR (film) ν_{max} = 3451, 2975, 2932, 1632, 1537, 1510, 1443, 1407, 1394, 1348, 1273, 1255, 1215, 1154, 1118, 1027, 931 cm^{-1}; ^1H NMR (400 MHz, CD_3OD): δ = 8.16 (d, *J* = 13.8 Hz, 1H), 7.88 (d, *J* = 10.1 Hz, 2H), 7.64 (s, 1H), 7.47 (d, *J* = 9.0 Hz, 1H), 7.33 (d, *J* = 8.2 Hz, 1H), 6.98–6.85 (m, 2H), 6.17 (d, *J* = 13.8 Hz, 1H), 4.35–4.21 (m, 2H), 3.54 (q, *J* = 7.0 Hz, 4H), 3.08–2.91 (m, 6H), 2.27–2.11 (m, 2H), 1.77 (s, 6H), 1.26 (t, *J* = 7.1 Hz, 6H); ^{13}C NMR (100 MHz, CD_3OD): δ = 173.3, 170.6, 157.8, 153.7, 144.9, 143.0, 142.1, 138.8, 136.9, 134.2, 131.2, 128.1, 124.6, 121.3, 115.6, 114.0, 111.2, 101.9, 98.1, 50.2, 46.2 (2C), 43.8, 30.8, 28.5 (2C), 26.2, 25.9, 23.8, 12.9 (2C) ppm; HPLC (system A): t_R = 4.1 min (purity 99% at 750 nm); LRMS (ESI+, recorded during RP-HPLC analysis): *m/z* 613.1 $[M + H]^+$ (100), calcd for $C_{31}H_{37}N_2O_7S_2^+$ 613.2; LRMS (ESI-, recorded during RP-HPLC analysis): *m/z* 611.2 $[M - H]^-$ (100), calcd for $C_{31}H_{35}N_2O_7S_2^-$ 611.2; HRMS (ESI+): *m/z* calcd for $C_{31}H_{37}N_2O_7S_2^+$ $[M + H]^+$ 613.2042, found 613.2044.



11: 72% yield; dark green solid; R_f = 0.5 (CH_2Cl_2/CH_3OH, 9:1, v/v); IR (film) ν_{max} = 3422, 2978, 2931, 2864, 1725, 1660, 1534, 1509, 1456, 1439, 1411, 1390, 1378, 1345, 1251, 1143, 1119, 1075, 1047, 1014, 927 cm^{-1}; ^1H NMR (400 MHz, CD_3OD): δ = 8.17 (d, *J* = 14.0 Hz, 1H), 7.57–7.44 (m, 2H), 7.41 (dd, *J* = 8.0, 3.6 Hz, 2H), 7.35–7.22 (m, 2H), 6.86 (m, *J* = 11.0 Hz, 2H), 5.98 (d, *J* = 14.1 Hz, 1H), 4.12 (t, *J* = 7.2 Hz, 2H), 3.53 (q, *J* = 7.0 Hz, 4H), 2.96 (dd, *J* = 28.0, 7.4 Hz, 4H), 2.31 (t, *J* = 7.1 Hz, 2H), 1.92–1.78 (m, 2H), 1.75 (s, 6H), 1.72–1.64 (m, 2H), 1.56–1.45 (m, 2H), 1.25 (t, *J* = 7.0 Hz, 6H); ^{13}C NMR (100 MHz, CD_3OD): δ = 177.4, 174.4, 169.7, 157.3, 153.2, 143.4, 142.6, 139.4, 135.0, 134.1, 130.9, 129.9, 126.6, 123.5, 122.5, 114.9, 113.3, 112.3, 101.8, 98.2, 50.7, 46.0 (2C), 45.0, 34.8, 28.4 (2C), 27.9, 27.4, 26.1, 25.8, 25.7, 12.9 (2C) ppm; HPLC (system A): t_R = 5.0 min (purity 96% at 750 nm); LRMS (ESI+, recorded during RP-HPLC analysis): *m/z* 525.2 $[M]^{+\circ}$ (100), calcd for $C_{34}H_{41}N_2O_3^+$ 525.3; HRMS (ESI+): *m/z* calcd for $C_{34}H_{41}N_2O_3^+$ $[M]^{+\circ}$ 525.3112, found 525.3132.



12: 68% yield; dark green solid; R_f = 0.66 (CH_2Cl_2/CH_3OH, 9:1, v/v); IR (film) ν_{max} = 3431, 2973, 2926, 1702, 1631, 1606, 1538, 1510, 1441, 1392, 1363, 1255, 1206, 1163, 1102, 1068, 1041, 1016, 939 cm^{-1}; ^1H NMR (400 MHz, CD_3OD): δ = δ 8.23 (d, *J* = 14.0 Hz, 1H), 8.07 (m, 2H), 7.62 (s, 1H), 7.47 (d, *J* = 9.5 Hz, 1H), 7.27 (d, *J* = 8.7 Hz, 1H), 6.94 (m, *J* = 3.9 Hz, 2H), 5.98 (d, *J* = 13.9 Hz, 1H), 3.68–3.52 (m, 7H), 3.11–2.92 (m, 4H), 1.78 (s, 6H), 1.37–1.19 (m, 6H); ^{13}C NMR (100 MHz, CD_3OD): δ = 174.7, 170.4, 157.8, 153.7, 146.6, 141.9, 139.0, 136.4, 134.1, 131.8, 131.1, 124.3, 123.7, 115.4, 113.8, 110.9, 101.7, 98.2, 50.1, 46.1 (2C), 31.7, 28.3 (2C), 26.0, 25.8, 12.8 (2C) ppm; HPLC (system A): t_R = 4.8 min (purity 99% at 750 nm); LRMS (ESI+, recorded during RP-HPLC analysis): *m/z* 469.2 $[M + H]^+$ (100), calcd for $C_{30}H_{33}N_2O_3^+$ 469.2; LRMS (ESI-, recorded during RP-HPLC analysis): *m/z* 467.4 $[M - H]^-$ (45) and 513.3 $[M - H + FA]^-$ (100), calcd for $C_{30}H_{31}N_3O_8S_2^-$ 467.2; HRMS (ESI+): *m/z* calcd for $C_{30}H_{33}N_2O_3^+$ $[M + H]^+$ 469.2486, found 469.2493.

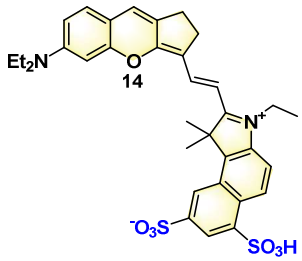


13: Carboxylic acid-functionalized *nor*-DHX-hemicyanine fused dye **12** (60 mg, 0.1 mmol, 1 eq.) was dissolved in dry DMF (2 mL). DIEA (34 L, 0.2 mmol, 2 eq.) and HATU (43 mg, 0.11 mmol, 1.1 eq.) were sequentially added. The resulting reaction mixture was stirred at 25 °C for 15 min. The resulting crude HOAt activated ester was added dropwise to a pre-cooled solution of 2-aminoethane-1,1-disulfonic acid (DIEA salt) in dry DMF (0.19 M in DMF, 1 mmol, 10 eq.) and DIEA (85 L, 0.5 mmol, 5 eq.) and the resulting mixture was stirred at 25 °C for 1 h. The reaction was checked for completion by TLC (CH_2Cl_2/CH_3OH, 9:1, v/v), quenched by adding glacial AcOH (50 L) and finally evaporated under reduced pressure. The resulting residue was dissolved in a (1:1, v/v) mixture of aq. 0.1% TFA and CH_3CN (ca. 5 mL) and purified by semi-preparative RP-HPLC (system B, t_R = 35.0–38.5 min). The product-containing fractions were lyophilized to give the TFA salt (1.5 TFA) of compound **13**, as a green amorphous powder (26 mg, 31%). R_f = 0.0 (CH_2Cl_2/CH_3OH, 9:1, v/v); IR (ATR): ν_{max} = 3417 (broad), 3068, 2981, 2939, 1657, 1631, 1614, 1548, 1499, 1456, 1401, 1358, 1273, 1228, 1178, 1114, 1058, 1039, 1013, 939, 909, 877, 803, 766, 734, 707, 651 cm^{-1}; ^1H NMR (500 MHz, [D_6]DMSO): δ = 1.19 (t, *J* = 7.5 Hz, 6H, 2 × C*H*$_3$-Et), 1.72 (s, 6H, 2 × C*H*$_3$), 2.94 (m, 4H, 2 × C*H*$_2$-DHX), 3.56 (m, 5H, -CH_2-C*H*(SO_3H)$_2$ & 2 × C*H*$_2$-Et), 3.66 (s, 3H, C*H*$_3$), 3.84 (bs, 2H, -C*H*$_2$-CH(SO_3H)$_2$), 6.00 (d, *J* = 13.5 Hz, 1H, C*H*-methine), 6.95 (bs, 2H), 7.48 (d, *J* = 8.5 Hz, 1H), 7.53 (d, *J* = 9.5 Hz, 1H), 7.74 (s + d, *J* = 9.5 Hz, 2H), 7.96 (s, 1H), 8.07 (d, *J* = 14.0 Hz, 1H, C*H*-methine), 8.48 (bs, 1H, N*H*) ppm; *To record the ^{13}C NMR spectrum, **13** was converted into TEA salt through a counter-ion exchange process by RP-HPLC (system C).* ^{13}C NMR (126 MHz, [D_6]DMSO): δ = 8.4 (*C*H_3-TEA), 12.5 (2C, *C*H_3-Et), 24.3, 24.8, 27.5 (2C, *C*H_2-Et), 31.4, 45.3, 45.6 (*C*H_2-TEA), 48.6, 74.1, 96.9, 101.1, 110.7,

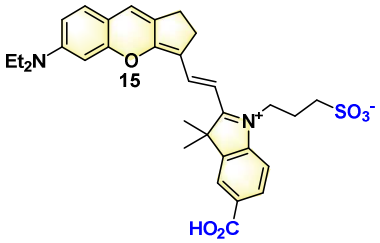
112.5, 113.5, 121.1, 122.0, 127.0, 129.9, 131.4, 132.5, 134.9, 136.8, 140.8, 145.0, 151.6, 155.7, 164.5, 168.0, 172.5 ppm; HPLC (system A): t_R = 4.3 min (purity 99% at 750 nm); ^{19}F NMR (282 MHz, [D$_6$]DMSO): δ = -73.5 (s, 3F, C*F*$_3$-TFA) ppm; LRMS (ESI+, recorded during RP-HPLC analysis): *m/z* 656.5 $[M + H]^+$ (100), calcd for $C_{32}H_{38}N_3O_8S_2^+$ 656.2; LRMS (ESI-, recorded during RP-HPLC analysis): *m/z* 654.3 $[M - H]^-$ (100), calcd for $C_{32}H_{36}N_3O_8S_2^-$ 654.2; HRMS (ESI+): *m/z* calcd for $C_{32}H_{38}N_3O_8S_2^+$ $[M + H]^+$ 656.20948, found 656.21059; elemental analysis (%) for $C_{32}H_{37}N_3O_8S_2$. 1.5 CF_3CO_2H: C 50.84, H 4.69, N, 5.08, S 7.76; found: 50.61, H 5.61, N, 5.29, S 7.27.

6) General procedure for the synthesis of water-soluble *nor*-DHX-hemicyanine fused dyes 14–18.

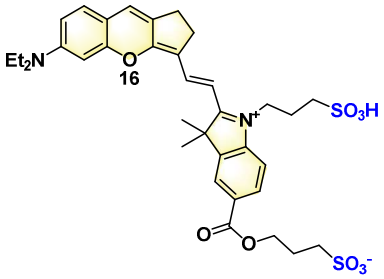
To *nor*-DHX aldehyde **3b** in HPLC-grade CH_3OH (0.025–0.05 M) were added the corresponding indolinium or benzoindolinium salt (1.2 eq.) and dry pyridine (one or two drops). The resulting reaction mixture was stirred under reflux (except for **18**, stirring at 25 °C) for 2 h and at 25 °C for 16 h (*please note*: the color gradually changed to green). The reaction was checked for completion by TLC (CH_2Cl_2/CH_3OH, 8:2, v/v), evaporated under reduced pressure and purified by flash-column chromatography on silica gel or by semi-preparative RP-HPLC (*vide infra*).



14: The crude product was purified by flash-column chromatography over silica gel (dry loading, step gradient of CH_3OH in CH_2Cl_2 from 0% to 20%). Bis-sulfonated *nor*-DHX-hemicyanine NIR dye **14** was obtained as a green solid (42 mg, yield 47%). R_f = 0.4 (CH_2Cl_2/CH_3OH, 85:15, v/v); IR (film) ν_{max} = 3466, 2978, 2933, 1632, 1541, 1507, 1452, 1414, 1393, 1377, 1353, 1313, 1271, 1253, 1211, 1167, 1144, 1111, 1067, 1022, 988 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD): δ = 9.04 (d, *J* = 9.2 Hz, 1H), 8.64 (d, *J* = 8.5 Hz, 2H), 8.23 (d, *J* = 14.1 Hz, 1H), 7.62 (d, *J* = 9.3 Hz, 1H), 7.39 (s, 1H), 7.27 (d, *J* = 8.9 Hz, 1H), 6.84–6.74 (m, 2H), 6.01 (d, *J* = 14.1 Hz, 1H), 4.24 (d, *J* = 7.2 Hz, 2H), 3.52 (d, *J* = 7.1 Hz, 4H), 2.96 (d, *J* = 9.8 Hz, 4H), 1.98 (s, 6H), 1.42 (t, *J* = 7.1 Hz, 3H), 1.24 (t, *J* = 7.1 Hz, 6H); ^{13}C NMR (100 MHz, CD_3OD): δ = 174.8, 169.9, 157.3, 153.3, 145.0, 144.1, 141.3, 138.6, 136.0, 135.9, 133.9, 131.1, 130.7, 129.4, 128.9, 123.2, 123.0 (2C), 115.0, 113.6, 113.2, 100.4, 97.8, 52.4, 46.1 (2C), 40.2, 28.4 (2C), 25.8, 25.8, 12.9, 12.6 (2C) ppm; HPLC (system A): t_R = 4.5 min (purity 99% at 750 nm); LRMS (ESI+, recorded during RP-HPLC analysis): *m/z* 649.3 $[M + H]^+$ (100), calcd for $C_{34}H_{37}N_2O_7S_2^+$ 649.2; LRMS (ESI-, recorded during RP-HPLC analysis): *m/z* 647.0 $[M - H]^-$calcd for $C_{34}H_{35}N_2O_7S_2^-$ 647.2; HRMS (ESI+): *m/z* calcd for $C_{34}H_{37}N_2O_7S_2^+$ $[M + H]^+$ 649.2037, found 649.2032.

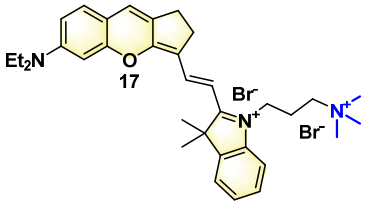


15: The crude product was dissolved in a (1:1, v/v) mixture of aq. 0.1% TFA and CH_3CN (ca. 5 mL) and purified by semi-preparative RP-HPLC (system B, t_R = 41.0–45.0 min). The product-containing fractions were lyophilized to give the TFA salt (1.5 TFA) of compound **15**, as a green amorphous powder (22 mg, 10%). *Please note*: the low isolated yield was explained by partial degradation of *nor*-DHX-hemicyanine fused dye in the crude reaction mixture due to too prolonged heating at reflux. R_f (CH_2Cl_2/CH_3OH, 8:2, v/v): 0.30; IR (ATR): ν_{max} = 2970, 2929, 2871, 1698, 1631, 1575, 1531, 1506, 1440, 1376, 1344, 1311, 1252, 1200, 1102, 1030, 932, 772, 726, 664 cm^{-1}; ^1H NMR (500 MHz, [D$_6$]DMSO): δ = 1.19 (t, J = 6.5 Hz, 6H, 2 × C$\underline{H}_3$-Et), 1.72 (s, 6H, 2 × C$\underline{H}_3$), 2.01 (bm, 2H), 2.50 (t, J = 6.0 Hz, 2H), 2.98 (bs, 4H, 2 × C$\underline{H}_2$-DHX), 3.55 (q, 4H, 2 × C$\underline{H}_2$-Et, partially masked by water signal), 4.34 (t, J = 7.0 Hz, 2H, $^+$N-C$\underline{H}_2$-$(CH_2)_2$-SO_3^-), 6.30 (bd, J = 13.5 Hz, 1H, C$\underline{H}$-methine), 6.99 (bs, 2H), 7.50 (d, J = 8.5 Hz, 1H), 7.56 (d, J = 9.0 Hz, 1H), 7.84 (s, 1H), 7.98 (d, J = 8.5 Hz, 1H), 8.05 (d, J = 13.5 Hz, 1H, C$\underline{H}$-methine), 8.10 (s, 1H) ppm; ^{13}C NMR (126 MHz, [D$_6$]DMSO): δ = 12.5 (2C, $\underline{C}H_3$-Et), 23.1, 24.4, 25.0, 27.7 (2C, $\underline{C}H_2$-Et), 44.4, 47.7, 48.3, 97.0, 101.3, 110.6, 112.9, 113.9, 123.3, 123.5, 126.3, 130.1, 130.6, 130.7, 132.7, 136.0, 136.7, 140.8, 145.9, 151.9, 156.0, 166.9, 168.5, 171.4 ppm; ^{19}F NMR (470 MHz, [D$_6$]DMSO): δ = -74.2 (s, 3F, C$\underline{F}_3$-TFA) ppm; HPLC (system A): t_R = 4.5 min (purity 99% at 750 nm); LRMS (ESI+, recorded during RP-HPLC analysis): *m/z* 577.1 $[M + H]^+$ (100) and 1153.2 $[2M + H]^+$ (5), calcd for $C_{32}H_{37}N_2O_6S^+$ 577.2; LRMS (ESI-, recorded during RP-HPLC analysis): *m/z* 574.8 $[M - H]^-$ (100) and 620.8 $[M - H + FA]^-$ (30), calcd for $C_{32}H_{35}N_2O_6S^-$ 575.2; HRMS (ESI+): *m/z* calcd for $C_{32}H_{37}N_2O_6S^+$ $[M + H]^+$ 577.23668, found 577.23706; elemental analysis (%) for $C_{32}H_{36}N_2O_6S$. 1.5 CF_3CO_2H: C 56.22, H 5.06, N, 3.75, S 4.29; found: 56.48, H 5.56, N, 3.83, S 4.21.

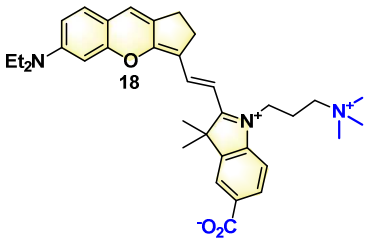


16: The crude product was purified by flash-column chromatography over silica gel column (dry loading, 20 × 170 mm, step gradient of CH_3OH in CH_2Cl_2 from 0% to 30%). Carboxylic acid 3-sulfonatopropyl ester-functionalized mono-sulfonated *nor*-DHX-hemicyanine fused dye **16** was obtained as a green solid which was submitted to a further purification by semi-preparative RP-HPLC (system B, t_R = 34.5–39.0 min). The product-containing fractions were lyophilized to give the TFA salt (1.25 TFA) of compound **16**, as a green amorphous powder (17 mg, 14%). R_f = 0.33 (CH_2Cl_2/CH_3OH, 8:2, v/v); IR (ATR): ν_{max} = 3348 (broad), 2930, 1704, 1632, 1578, 1542, 1455, 1429, 1407, 1371, 1352, 1314, 1273, 1223, 1178, 1134, 1028, 938, 870, 843, 774, 726 cm^{-1}; ^1H NMR (600 MHz, [D$_6$]DMSO): δ = 1.18 (t, J = 6.6 Hz, 6H, 2 × C$\underline{H}_3$-Et), 1.72 (s, 6H, 2 × C$\underline{H}_3$), 1.97-2.07 (m, 4H), 2.60 (m, 4H), 2.98 (bm, 4H, 2 × C$\underline{H}_2$-DHX), 3.54 (q, J = 6.6 Hz, 4H, 2 × C$\underline{H}_2$-Et), 4.30-4.40 (m, 4H, O-C$\underline{H}_2$-$(CH_2)_2$-SO_3H & $^+$N-C$\underline{H}_2$-$(CH_2)_2$-SO_3^-), 6.28 (bd, J = 13.2 Hz, 1H, C$\underline{H}$-methine), 6.97-6.99 (bm, 2H), 7.47 (d, J =

8.4 Hz, 1H), 7.56 (d, *J* = 9.6 Hz, 1H), 7.85 (s, 1H), 7.98 (dd, *J* = 8.4, 1.2 Hz, 1H), 8.02 (d, *J* = 13.8 Hz, 1H, C*H*-methine), 8.09 (bd, *J* = 1.2 Hz, 1H) ppm; ^{13}C NMR (151 MHz, [D$_6$]DMSO): δ = 12.5 (2C, *C*H$_3$-Et), 23.0, 24.5, 24.9, 25.0, 27.7 (2C, *C*H$_2$-Et), 44.4, 47.7, 47.9, 48.2, 64.0, 97.0, 101.2, 110.5, 113.0, 114.0, 123.0, 123.7, 125.3, 130.1, 130.5, 132.6, 136.2, 136.4, 140.8, 146.1, 151.9, 156.0, 165.4, 168.5, 171.0 ppm; ^{19}F NMR (282 MHz, [D$_6$]DMSO): δ = -75.2 (s, 3F, C*F*$_3$-TFA) ppm; HPLC (system A): t_R = 4.3 min (purity 100% at 750 nm); LRMS (ESI+, recorded during RP-HPLC analysis): *m/z* 699.6 [M + H]$^+$ (100), calcd for $C_{35}H_{43}N_2O_9S_2^+$ 699.2; LRMS (ESI-, recorded during RP-HPLC analysis): *m/z* 697.4 [M - H]$^-$ (100), calcd for $C_{35}H_{41}N_2O_9S_2^-$ 697.2; HRMS (ESI+): *m/z* calcd for $C_{35}H_{43}N_2O_9S_2^+$ [M + H]$^+$ 699.24045, found 699.24151; elemental analysis (%) for $C_{35}H_{42}N_2O_9S_2$. 1.25 CF_3CO_2H: C 53.53, H 5.18, N 3.33, S 7.62; found: C 53.74, H 6.13, N 3.59, S 7.44.



17: The crude product was purified by flash-column chromatography over silica gel column (dry loading, step gradient of CH_3OH in CH_2Cl_2 from 0% to 50%). *N*-(Trimethylammonio)propyl *nor*-DHX-hemicyanine NIR dye **17** was obtained as a green solid (44 mg, 33%). R_f = 0.33 (CH_2Cl_2/CH_3OH, 9:1, v/v); IR (film) ν_{max} = 3436, 1633, 1540, 1510, 1479, 1458, 1444, 1410, 1390, 1348, 1256, 1218, 1190, 1167, 1139, 1119, 1052, 1020, 924 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD): δ = 8.24 (d, *J* = 13.8 Hz, 1H), 7.69 (s, 1H), 7.59–7.42 (m, 4H), 7.31 (t, *J* = 7.4 Hz, 1H), 7.02–6.91 (m, 2H), 6.14 (d, *J* = 13.8 Hz, 1H), 4.33 (t, *J* = 7.6 Hz, 2H), 3.91–3.82 (m, 2H), 3.62 (q, *J* = 7.5, 7.1 Hz, 4H), 3.37 (s, 3H), 3.28–3.24 (m, 6H), 3.12 (d, *J* = 7.2 Hz, 4H), 2.47–2.32 (m, 2H), 1.84 (s, 6H), 1.32 (t, *J* = 7.0 Hz, 6H); ^{13}C NMR (100 MHz, CD_3OD): δ = 173.5, 170.4, 157.6, 153.5, 143.2, 142.3, 139.1, 136.5, 134.1, 131.2, 129.9, 126.2, 123.9, 123.5, 115.4, 113.8, 112.1, 101.4, 98.2, 64.5, 54.0, 53.8, 50.5, 46.1 (2C), 41.7, 30.7, 28.7 (2C), 26.7, 25.9, 22.2, 12.9 (2C) ppm; HPLC (system A): t_R = 4.1 min (purity 99%); LRMS (ESI+, recorded during RP-HPLC analysis): *m/z* 255.9 [M]$^{2+\circ}$ (100), calcd for $C_{34}H_{45}N_3O^{2+}$ 511.3; LRMS (ESI-, recorded during RP-HPLC analysis): *m/z* 600.4 [M - 3H + 2FA]$^-$ (100) and 646.4 [M - 3H + 3FA]$^-$ (40), calcd for $C_{34}H_{42}N_3O^-$ 508.3; HRMS (ESI+): *m/z* calcd for $C_{34}H_{45}N_3O_2^+$ [M]$^{2+\circ}$ 255.6776, found 255.6776.



18: The crude product was dissolved in a (1:1, v/v) mixture of aq. 0.1% TFA and CH_3CN (ca. 5 mL) and purified by semi-preparative RP-HPLC (system B, t_R = 32.0–41.0 min). The product-containing fractions were lyophilized to give the TFA salt (3.5 TFA) of compound **18**, as a green amorphous powder (60 mg, 37%). IR (ATR): ν_{max} = 2977, 2935, 1686, 1666, 1632, 1577, 1533, 1507, 1440, 1376, 1348, 1313, 1274, 1252, 1205, 1105, 1040, 933, 820, 772, 707, 664 cm^{-1}; ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 1.21 (t, *J* = 7.0 Hz, 6H, 2 × C*H*$_3$-Et), 1.75 (s, 6H, 2 × C*H*$_3$), 2.15 (bm, 2H), 2.95-3.05 (m, 4H, 2 × C*H*$_2$-DHX), 3.09 (s, 9H, $^+$N-C*H*$_3$), 3.50 (qt, 2H, $^+$N-CH$_2$-C*H*$_2$-CH$_2$-$^+$N(CH$_3$)$_3$), 3.60 (q, *J* = 7.0 Hz, 4H, 2 × C*H*$_2$-Et), 4.16 (t, *J* = 7.0 Hz, 2H, $^+$N-C*H*$_2$-(CH$_2$)$_2$-$^+$N(CH$_3$)$_3$), 5.96 (d, *J* = 15.0 Hz, 1H, C*H*-methine), 7.06-

7.10 (m, 2H), 7.45 (d, J = 8.5 Hz, 1H), 7.65 (d, J = 9.0 Hz, 1H), 8.0 (s+d+d, 3H, 2 × Ar-*H*, C*H*-methine), 8.12 (s, 1H) ppm; ^{13}C NMR (126 MHz, [D$_6$]DMSO): δ = 12.5 (2C, *C*H$_3$-Et), 20.4, 24.4, 24.8, 27.8 (2C, *C*H$_2$-Et), 40.3, 44.5, 48.0, 52.4 (3C), 62.5, 96.8, 99.9, 110.0, 113.7, 114.6, 123.4, 124.0, 126.2, 130.5 (2C), 132.3, 135.4, 137.7, 140.4, 145.9, 152.4, 156.4, 166.9, 168.9, 169.8 ppm; ^{19}F NMR (470 MHz, [D$_6$]DMSO): δ = -74.1 (s, 3F, C*F*$_3$-TFA) ppm; HPLC (system A): t_R = 3.8 min (purity 98%); LRMS (ESI+, recorded during RP-HPLC analysis): *m/z* 277.6 $[M]^{2+\circ}$ (100), calcd for $C_{35}H_{45}N_3O_3^{2+}$ 555.3; LRMS (ESI-, recorded during HPLC analysis): *m/z* 597.8 $[M^{2+\circ}$ - 3H + FA$]^-$ (35), 644.0 $[M^{2+\circ}$ - 3H + 2FA$]^-$ (100), 689.9 $[M^{2+\circ}$ - 3H + 3FA$]^-$ (55), calcd for $C_{35}H_{42}N_3O_3^-$ 552.3; HRMS (ESI+): *m/z* calcd for $C_{35}H_{45}N_3O_3^{2+}$ $[M]^{2+\circ}$ 277.67250, found 277.67360; elemental analysis (%) for $C_{35}H_{45}N_3O_3$. 2 $CF_3CO_2^-$. 1.5 CF_3CO_2H: C 53.00, H 4.82, N 4.41; found C 53.19, H 5.38, 4.56.

7) Preparation of fluorescent BSA conjugates.

(a) Synthesis of NHS esters: carboxylic acid-functionalized *nor*-DHX-hemicyanine fused dye **12**, **15** or **18** (1.2–2.0 mole, 1 eq., weighed in a 0.5 mL "eppendorf"-type microtube) was dissolved in dry DMSO (final concentration 25 mM). 1.1 eq. of TSTU (7.6–9.0 L of a 180 mM solution in DMSO) and 2 (or 3 for **18**) eq. of DIEA (1.47–2.0 L of a 2.0 M solution in NMP) were sequentially added and the resulting mixture was periodically vortexed for 1 h. The reaction was checked for completion by ESI-MS. The resulting NHS esters were used in the next BSA labeling step without purification.

*NHS ester of **12***: LRMS (ESI+): *m/z* 566.3 $[M + H]^+$, calcd for $C_{34}H_{36}N_3O_5^+$ 566.2.
*NHS ester of **15***: LRMS (ESI+): *m/z* 674.2 $[M + H]^+$, calcd for $C_{36}H_{40}N_3O_8S^+$ 674.2.
*NHS ester of **18***: LRMS (ESI+): *m/z* 669.3 $[M^{2+\circ} + H_2O - H]^+$, calcd for $C_{39}H_{47}N_4O_5^+$ 651.3. *Addition of a water molecule was occurred during the ionization*.

(b) Fluorescent labeling of BSA: The solution of NHS ester (see above, 15- or 30-fold excess according to protein) was added to a solution of BSA (500 L, 1.8 mg/mL, 13.5 nmol) in phosphate buffer (pH 7.05 or 7.70). The resulting mixture was protected from light and periodically vortexed. The reaction was left at 4 °C overnight and further 2 h at 20 °C. Thereafter, the mixture was diluted with phosphate buffer (1 mL), centrifugated to remove insoluble materials (excess of NHS ester and/or starting dye). Thereafter, the solution was transferred to an ultra-centrifugal filter device (Amicon Ultra 2 mL, Ultracel cut-off 30 kDa from Merck Millipore, ref. UFC203024) and centrifugated at 4000 rpm for 15 min. For each fluorescent BSA conjugate, 50–100 L of solution were recovered. Confirmation of conjugation to the protein was achieved by MALDI-TOF mass spectrometry.

(c) Gel analysis of conjugates: As a quality control for each conjugate, SDS-PAGE were performed using a ECLTM Gel Box system (GE Healthcare). Each conjugate was separated by SDS-PAGE with a 4–20% gradient ECL Gel (GE healthcare) under non-reducing conditions (loading buffer: 50 mM Tris.HCl (pH 6.8), 1% SDS, 0.1% bromophenol blue and 15% glycerol). After electrophoresis at 160 V for 1 h, the gel was imaged by fluorescence scanning with IVIS Lumina III in vivo imaging system (Perkin Elmer, Ex/Em filters 660/790 nm, bandwith 20/40 nm).

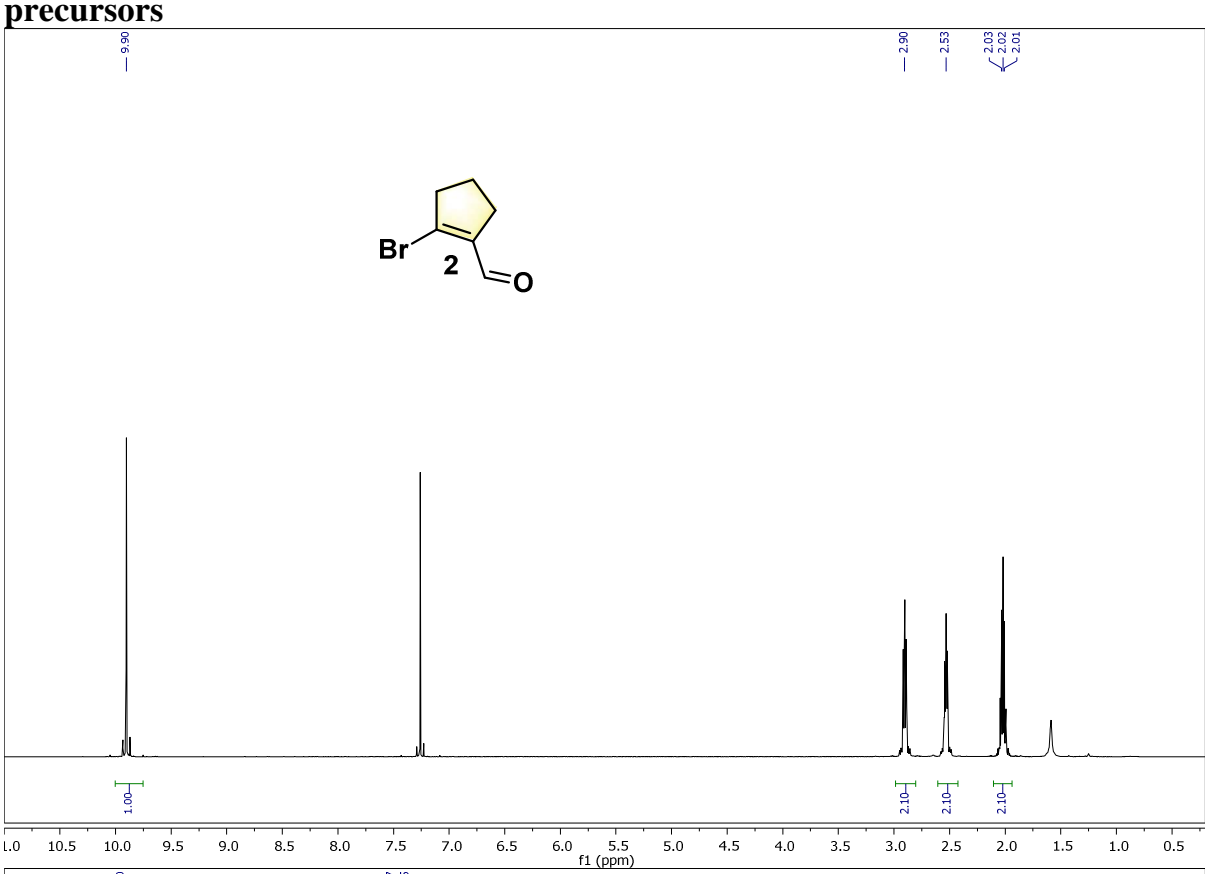
(d) Determination of dye-to-protein ration by spectrophotometric method:
[F/P] of these conjugates were determined spectrophotometrically by measuring their absorbance at 280 nm and 728 nm (λ_{max} of the *nor*-DHX-hemicyanine fused dye) and inserting the measured values into the following equation:

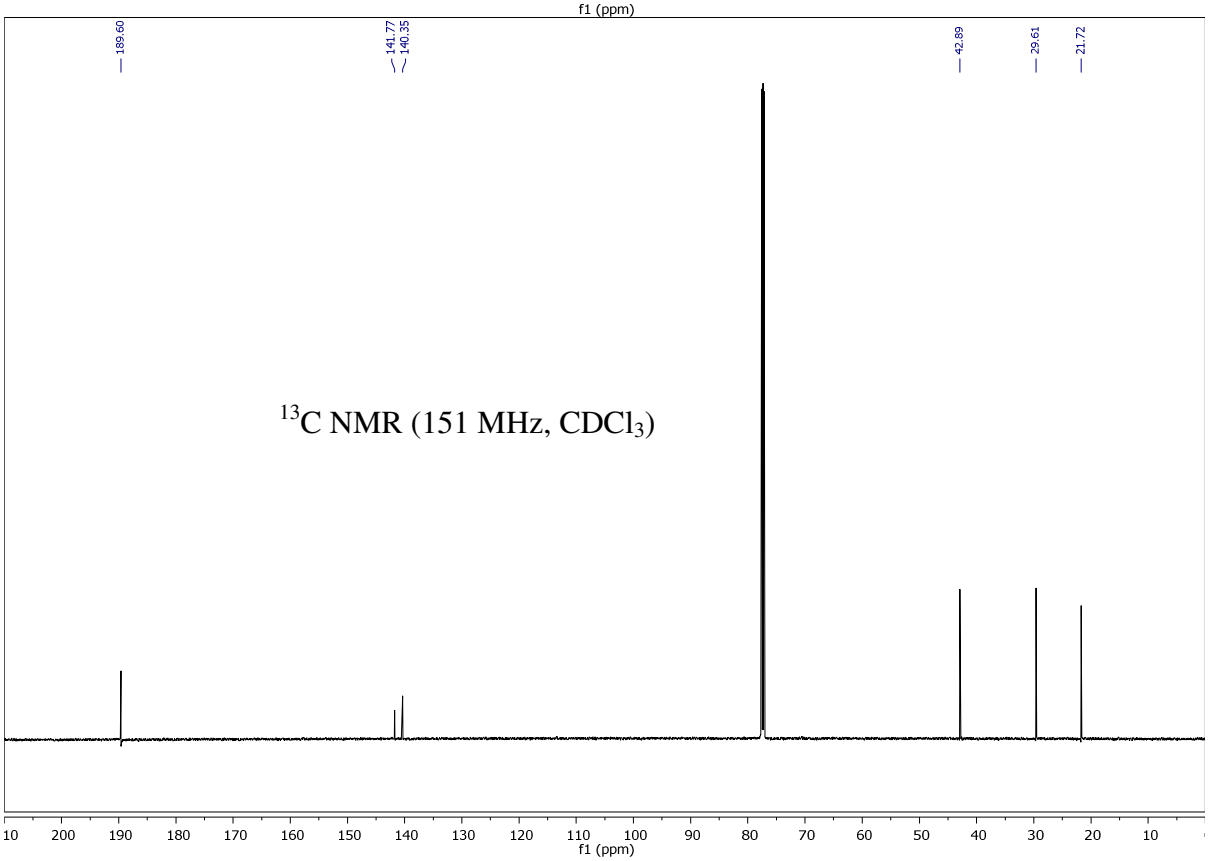
$$[F/P] = A_{max} P_{\varepsilon 280} / (A_{280} F_{\varepsilon max} - A_{max} F_{\varepsilon 280})$$

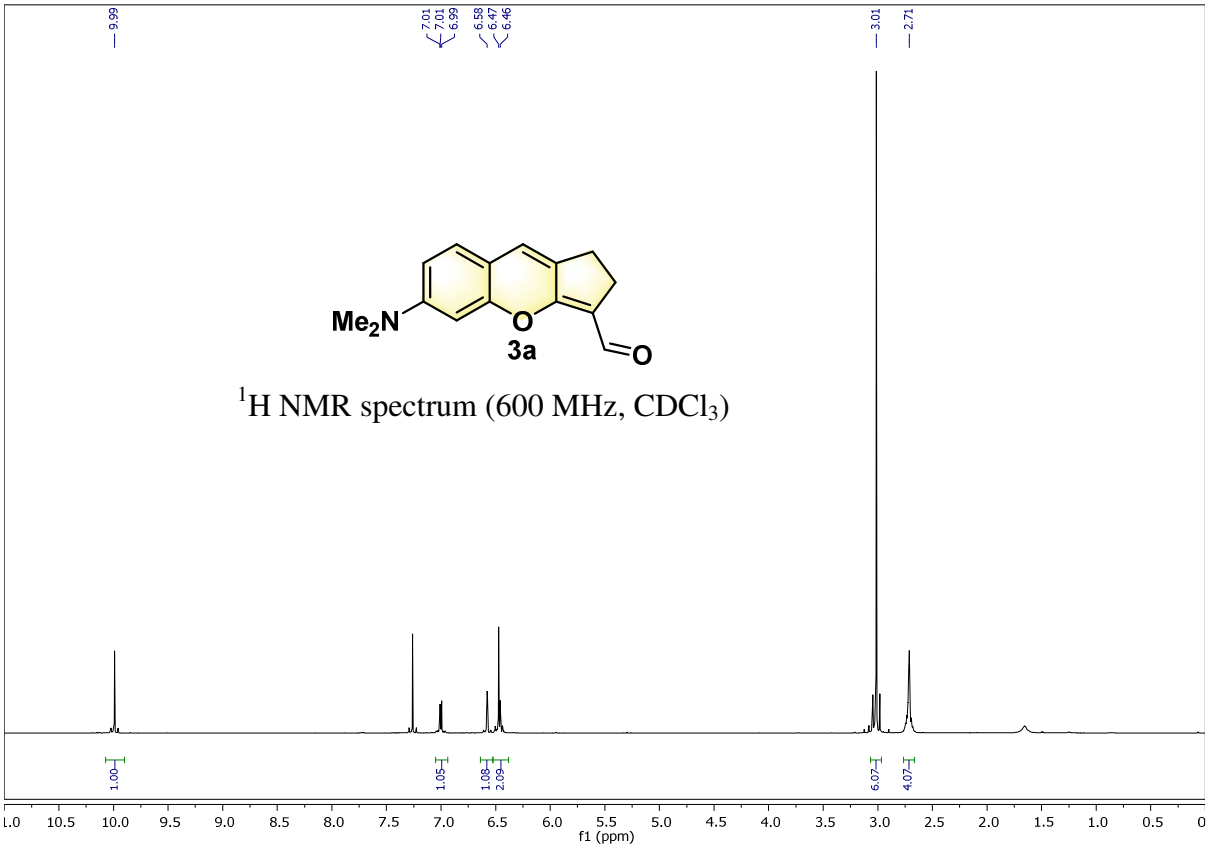
Where A_{280} is the absorbance of the protein at 280 nm, $P_{\varepsilon 280}$ is the extinction coefficient of the BSA protein at 280 nm (43 824 M^{-1} cm^{-1}), A_{max} is the absorbance of the DHX-hemicyanine label at its absorption maximum, $F_{\varepsilon max}$ is the extinction coefficient of the free unbound fluorophore at the absorption maximum (129 180 M^{-1} cm^{-1} for **12**, 137 240 M^{-1} cm^{-1} for **15** and 158 075 M^{-1} cm^{-1} for **18**), and $F_{\varepsilon 280}$ is the extinction coefficient of the free unbound fluorophore at 280 nm (13 720 M^{-1} cm^{-1} for **12**, 14 670 M^{-1} cm^{-1} for **15** and 15 660 M^{-1} cm^{-1} for **18**). We used a correction procedure for the visible part of the spectra at high [F/P] ratios. Specifically, we considered the integrated area under the visible part of the spectrum (and not the height of the dye peak) for quantization of the dye in solution. We assumed that the quantity of the dye is proportional to the integrated visible absorption spectrum or area under the curve (AUC) from 550 to 850 nm, and calculated a correction factor for each conjugate equal to the ratio of the AUC for the conjugate to the AUC for the free nonbound label (normalized spectra).

Analytical data of the synthesized compounds

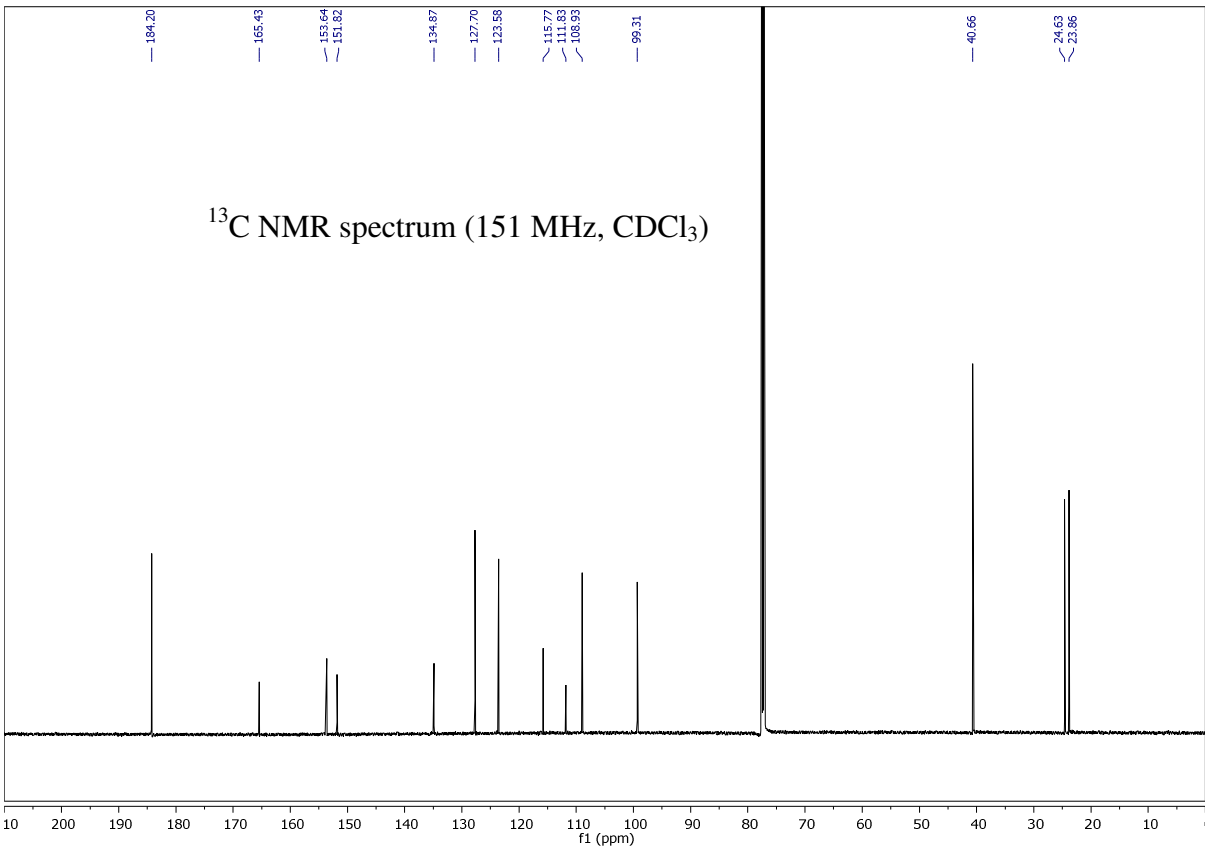
1) ^{1}H and ^{13}C NMR spectra of *nor*-DHX-hemicyanine fused NIR dyes and precursors



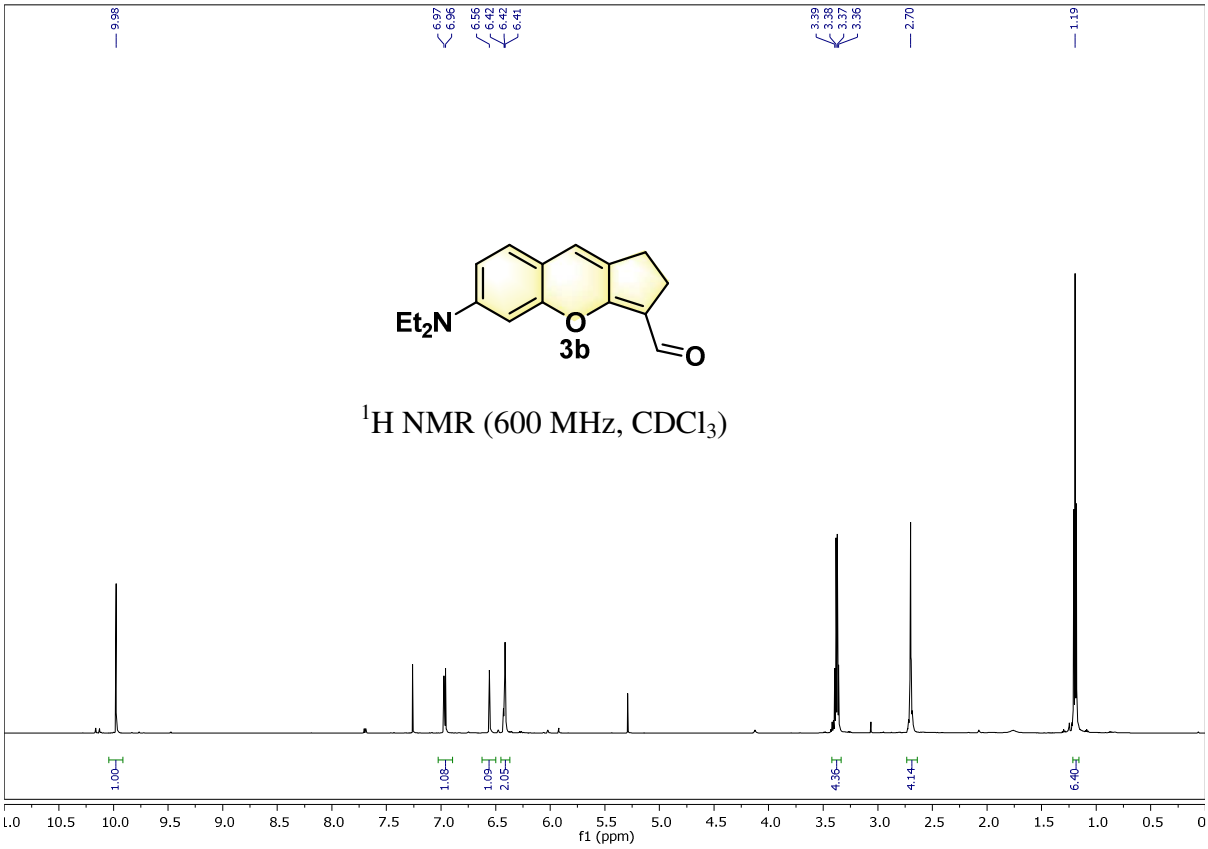




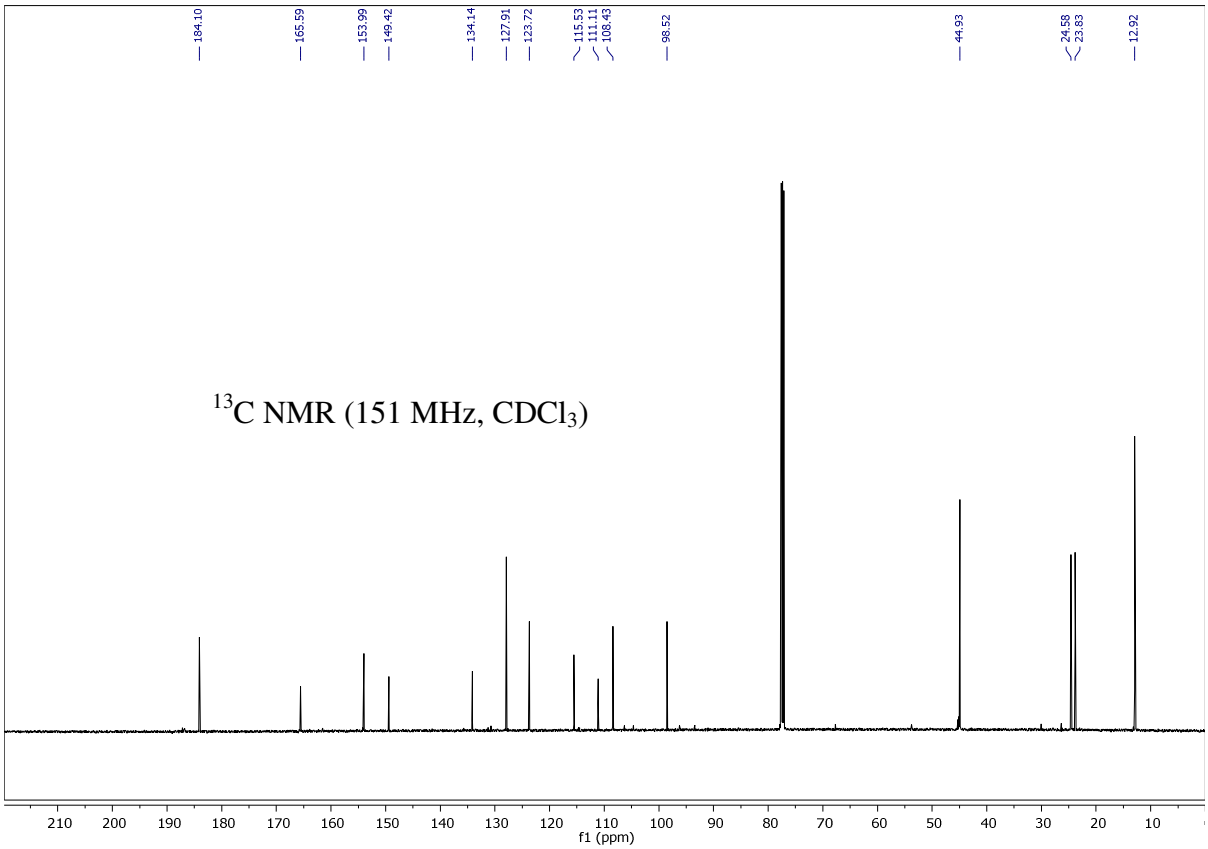
^{1}H NMR spectrum (600 MHz, $CDCl_3$)



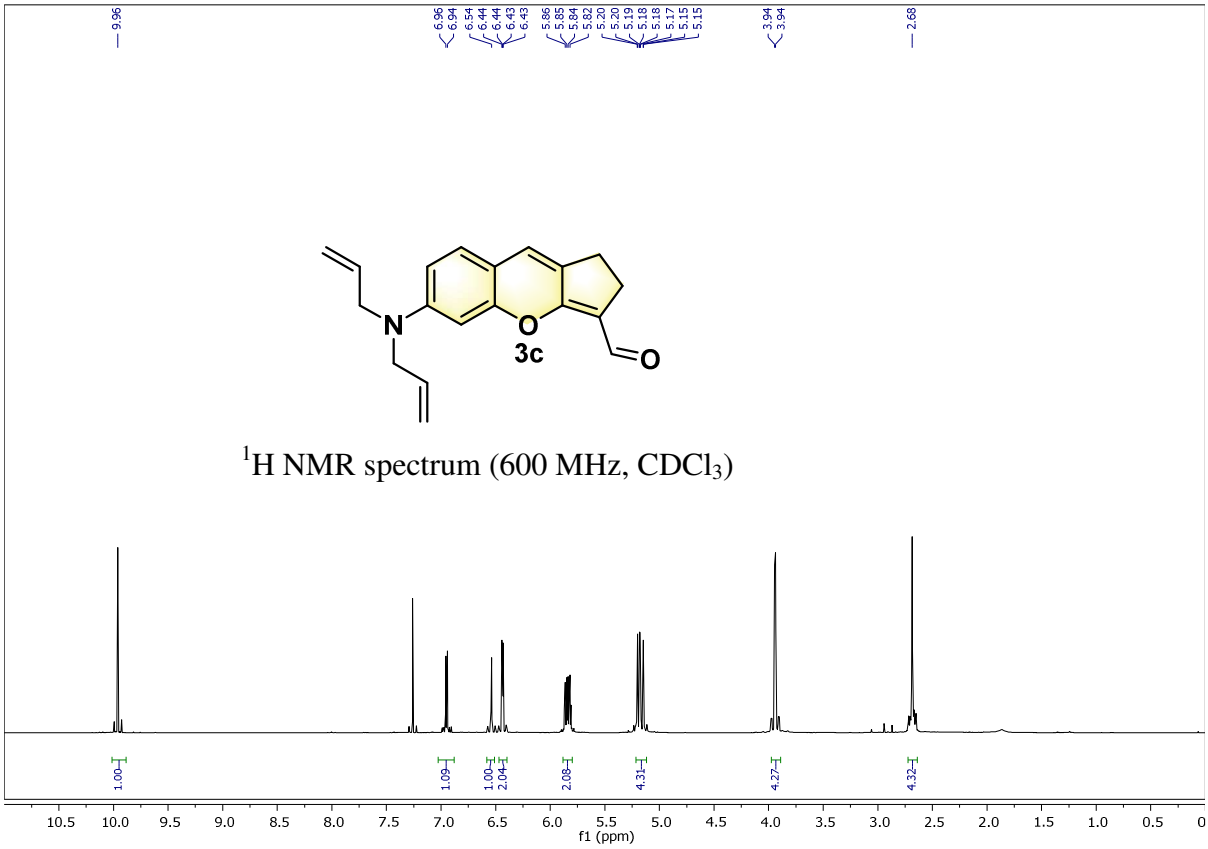
^{13}C NMR spectrum (151 MHz, $CDCl_3$)



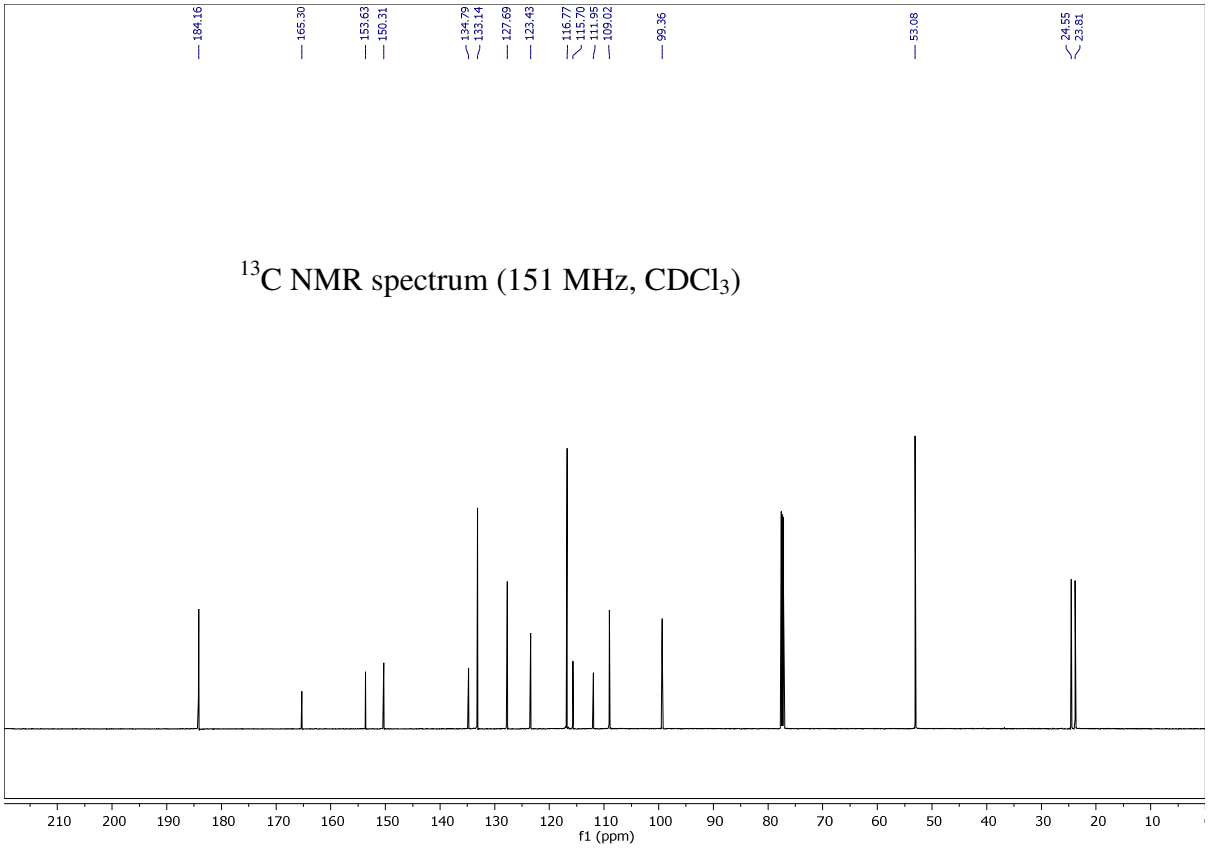
^{1}H NMR (600 MHz, $CDCl_3$)



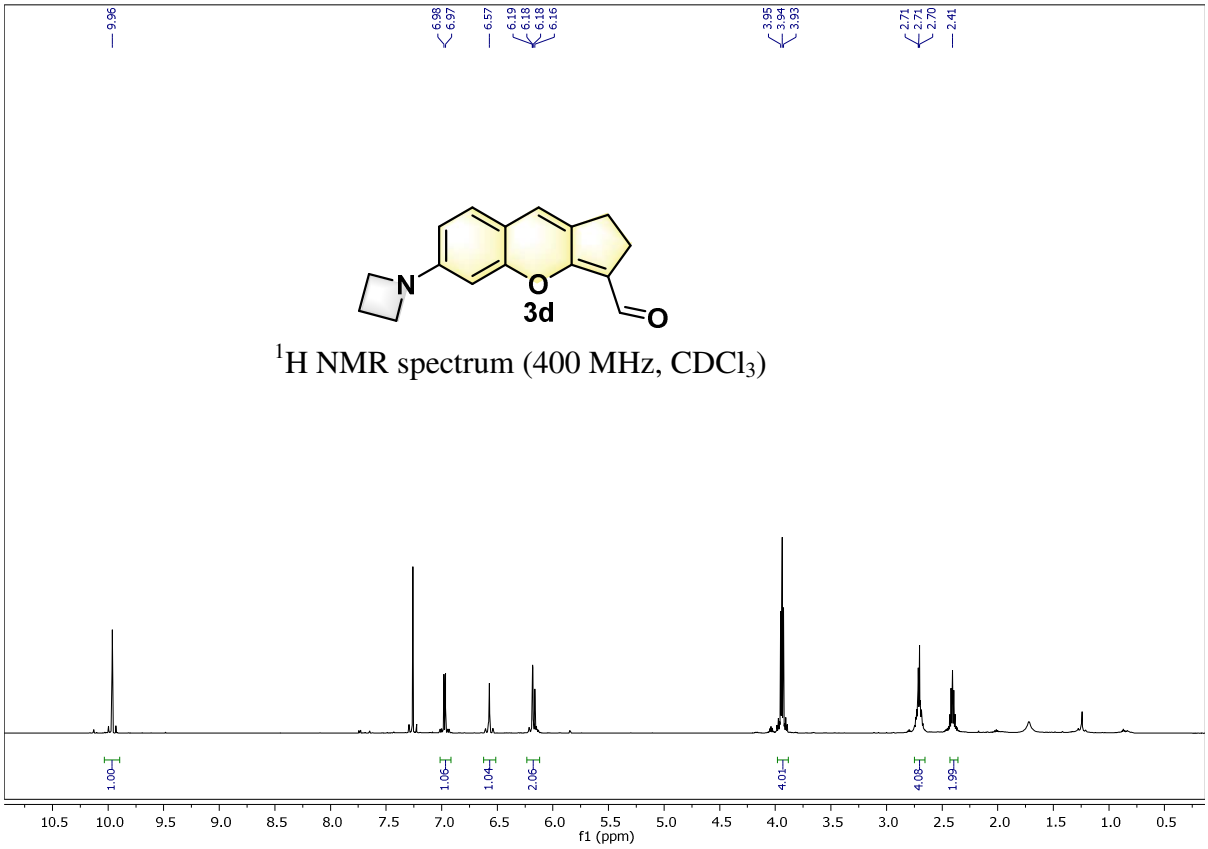
^{13}C NMR (151 MHz, $CDCl_3$)



^{1}H NMR spectrum (600 MHz, $CDCl_3$)



^{13}C NMR spectrum (151 MHz, $CDCl_3$)



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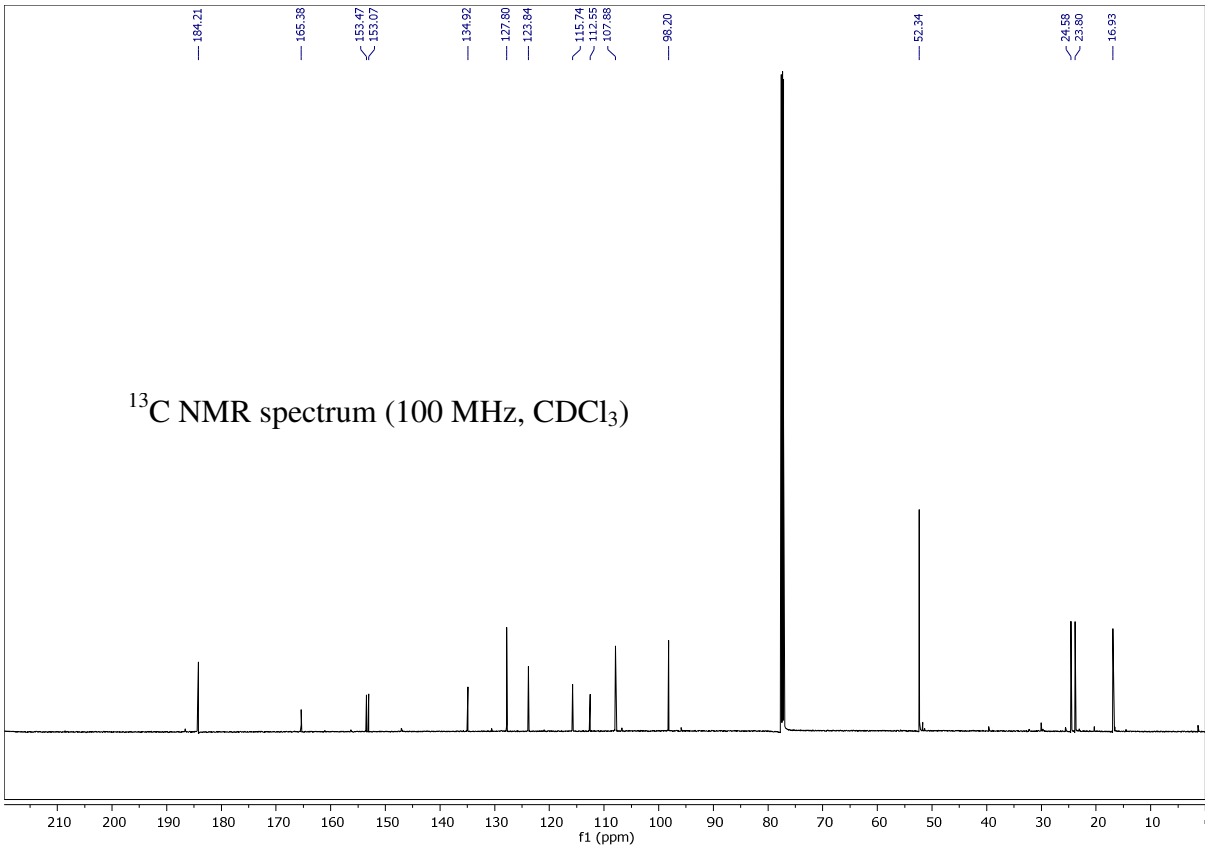

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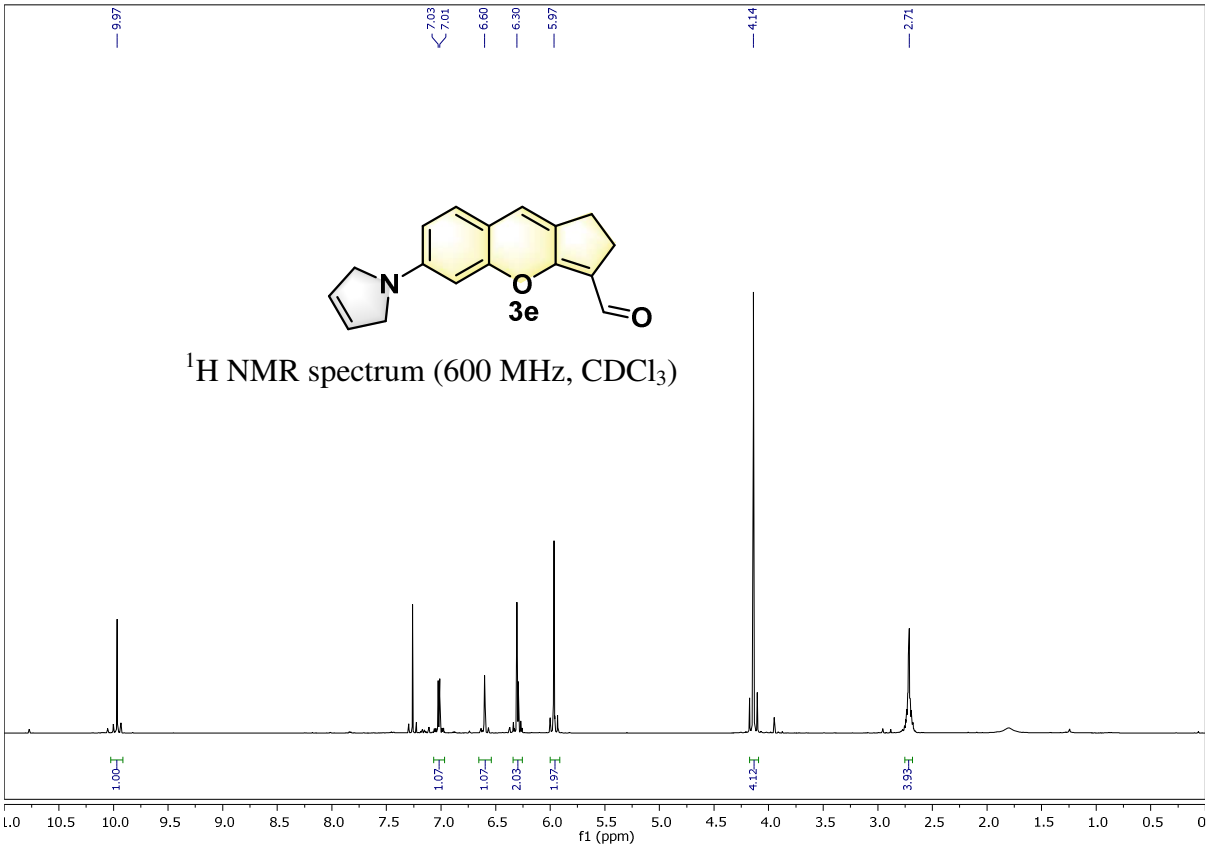

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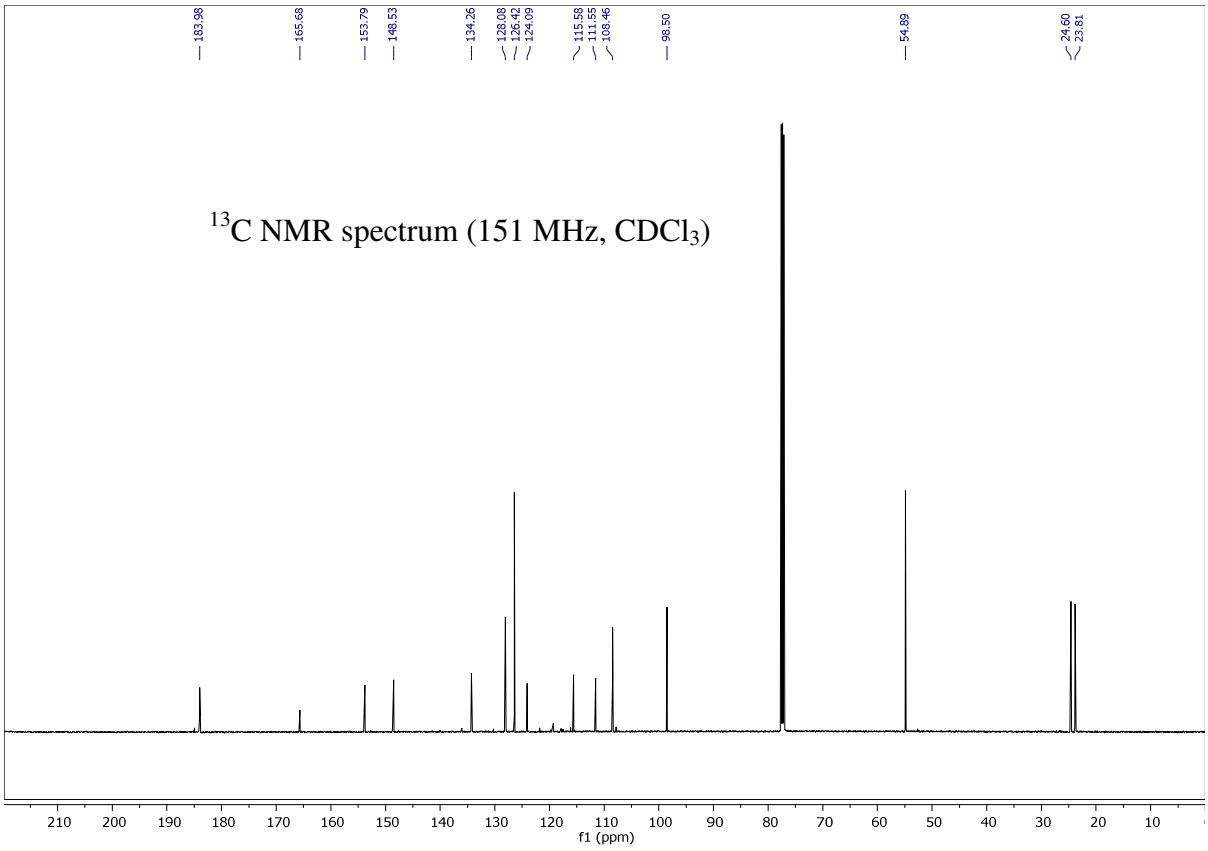
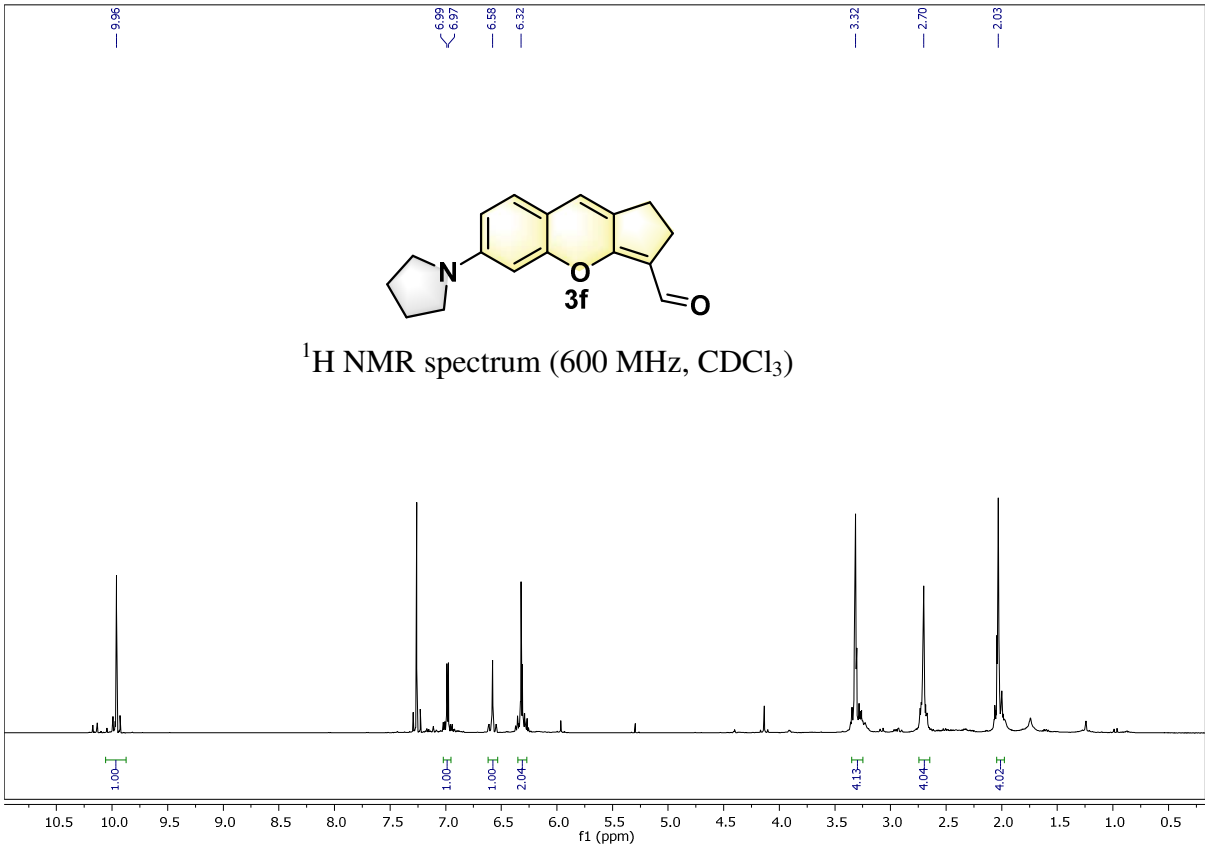
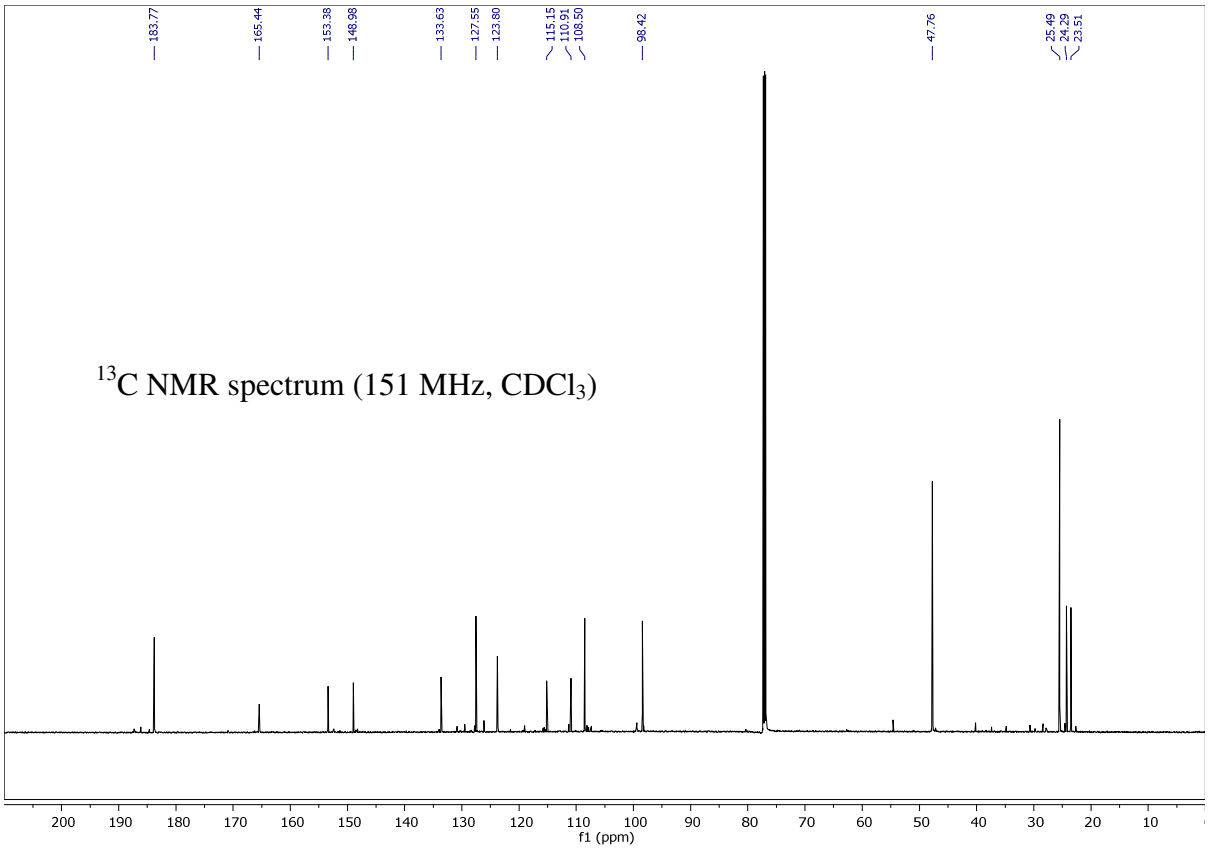


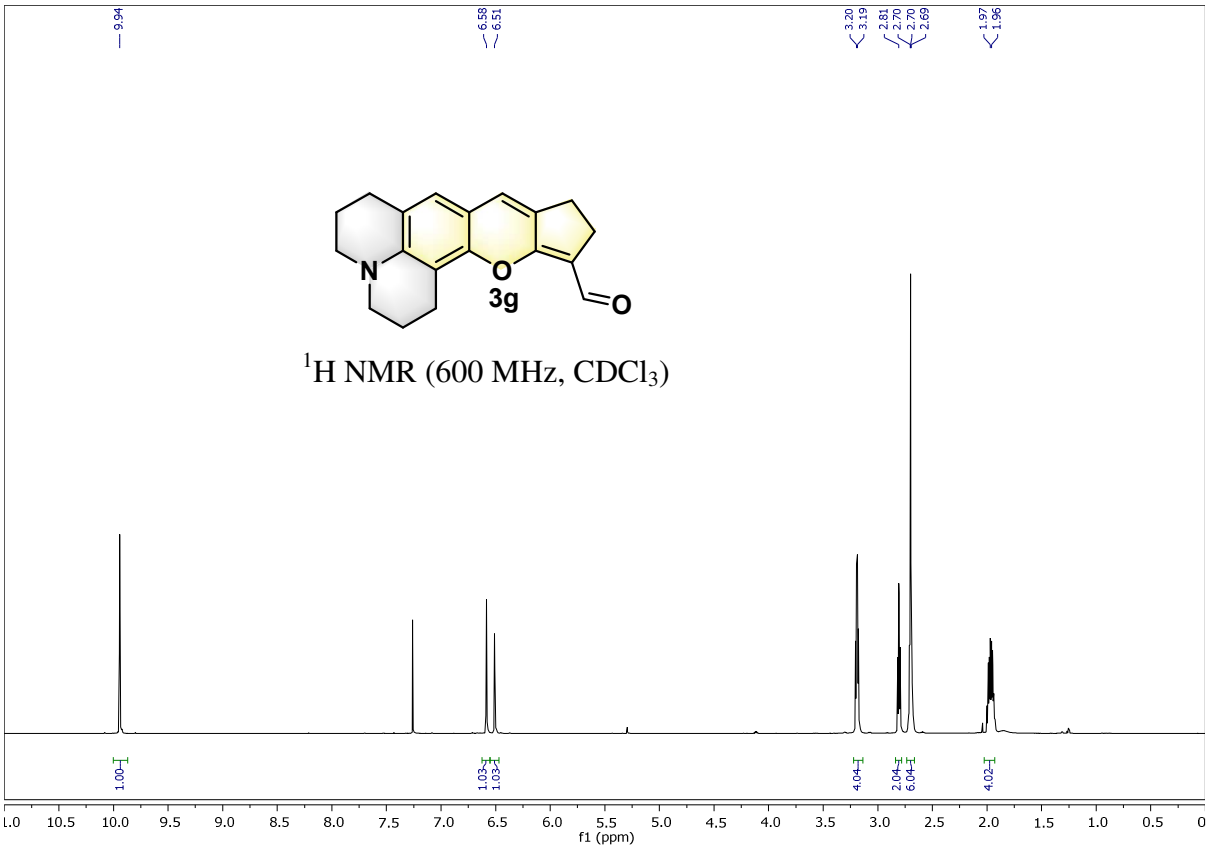
^{13}C NMR spectrum (151 MHz, $CDCl_3$)



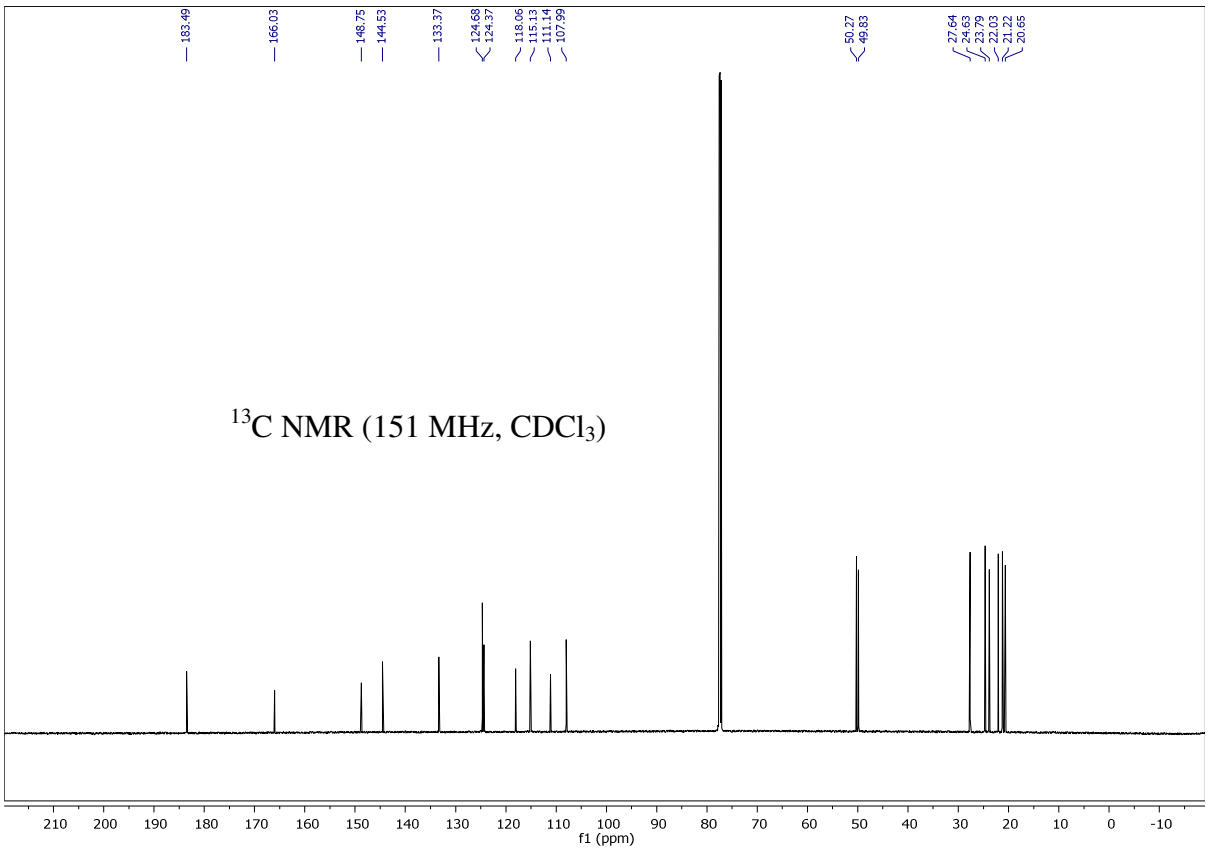
^{1}H NMR spectrum (600 MHz, $CDCl_3$)



^{13}C NMR spectrum (151 MHz, $CDCl_3$)



^{1}H NMR (600 MHz, $CDCl_3$)



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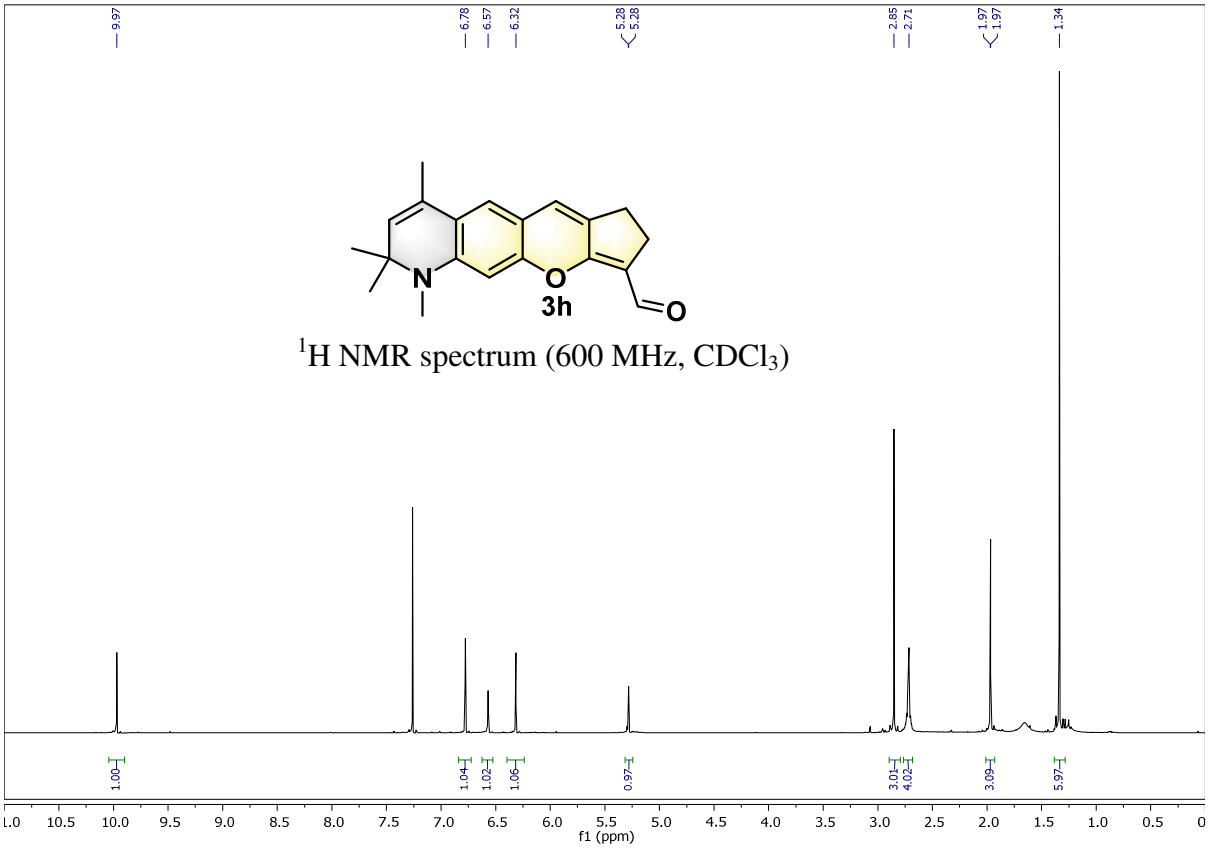

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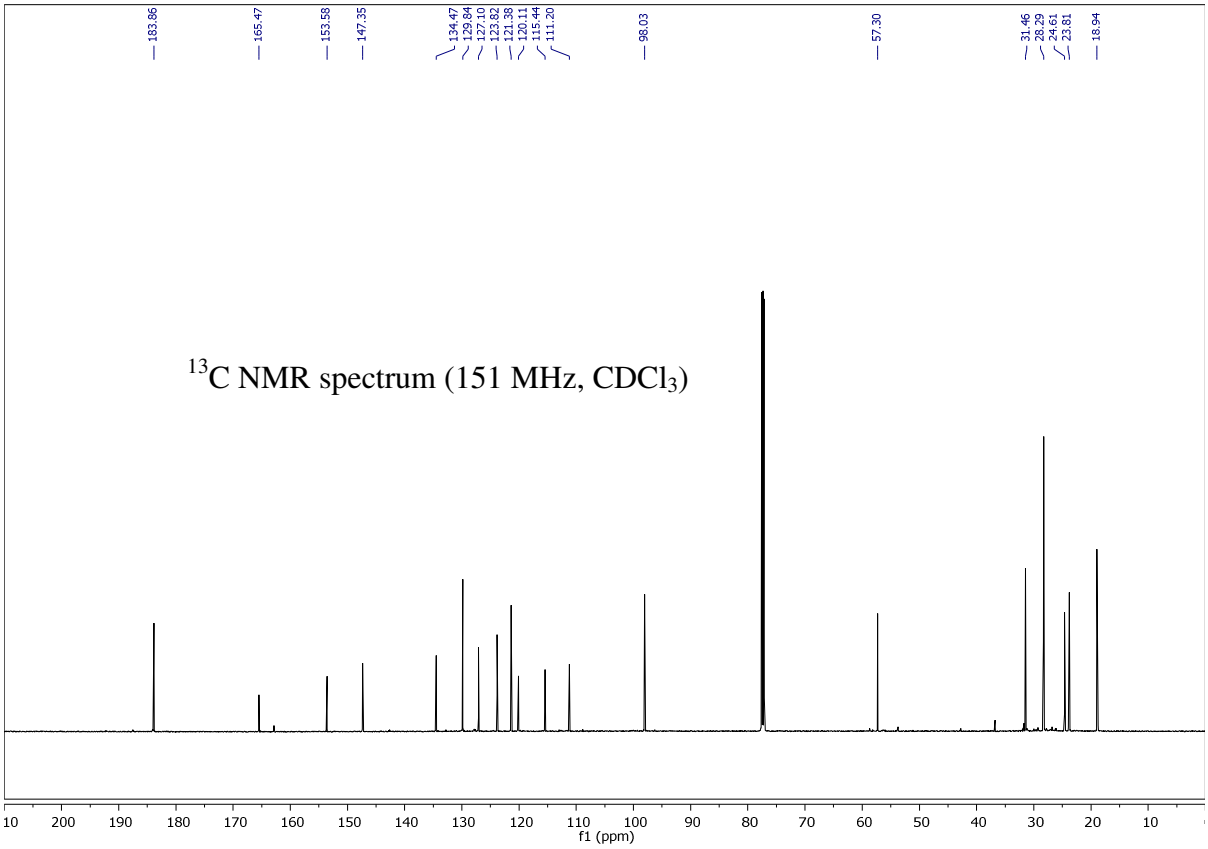
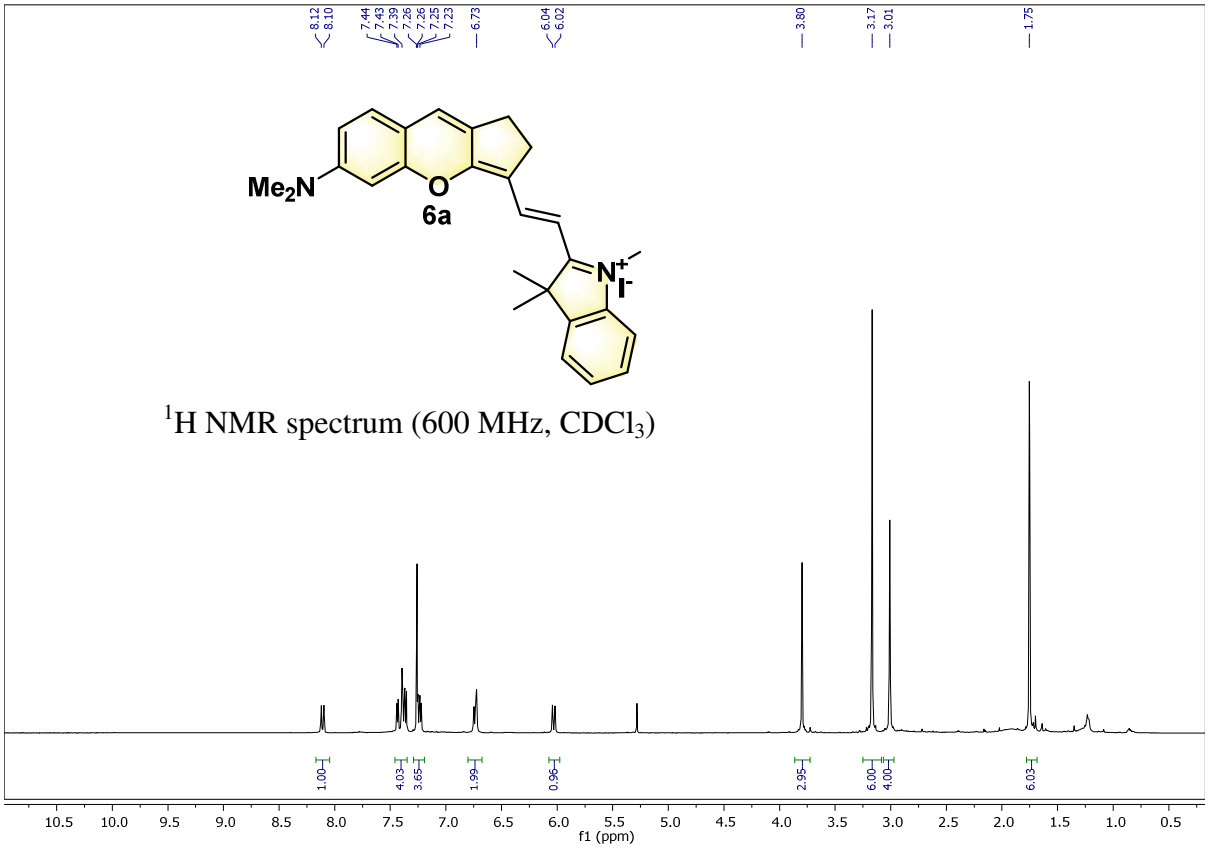


^{13}C NMR spectrum (151 MHz, $CDCl_3$)



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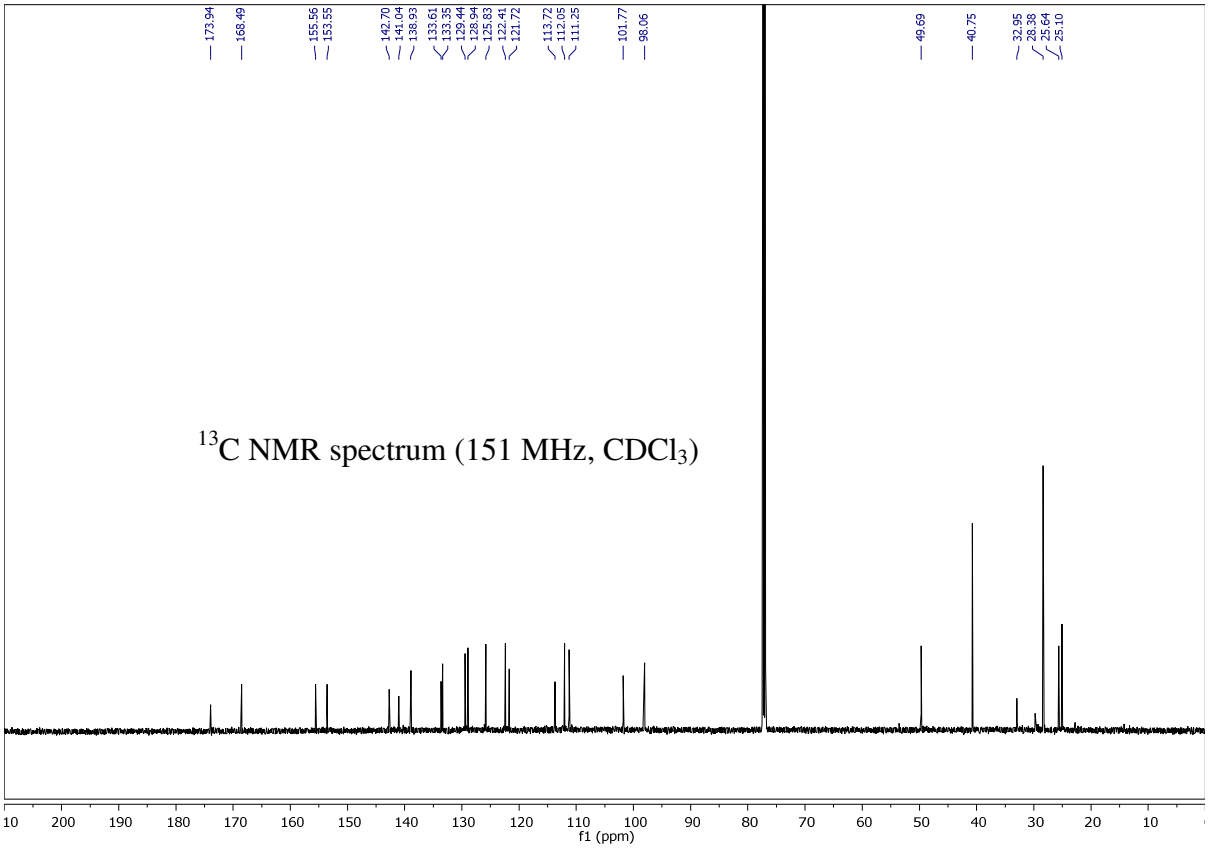

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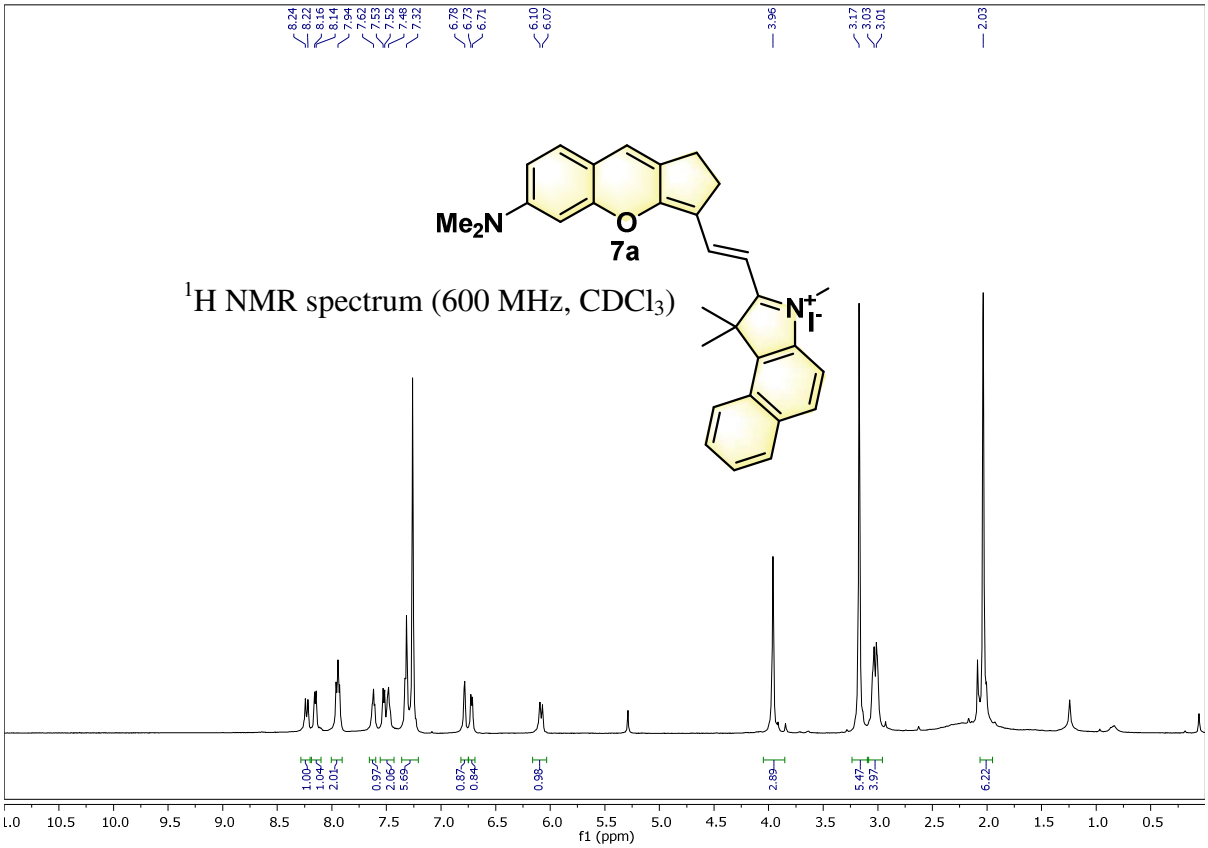

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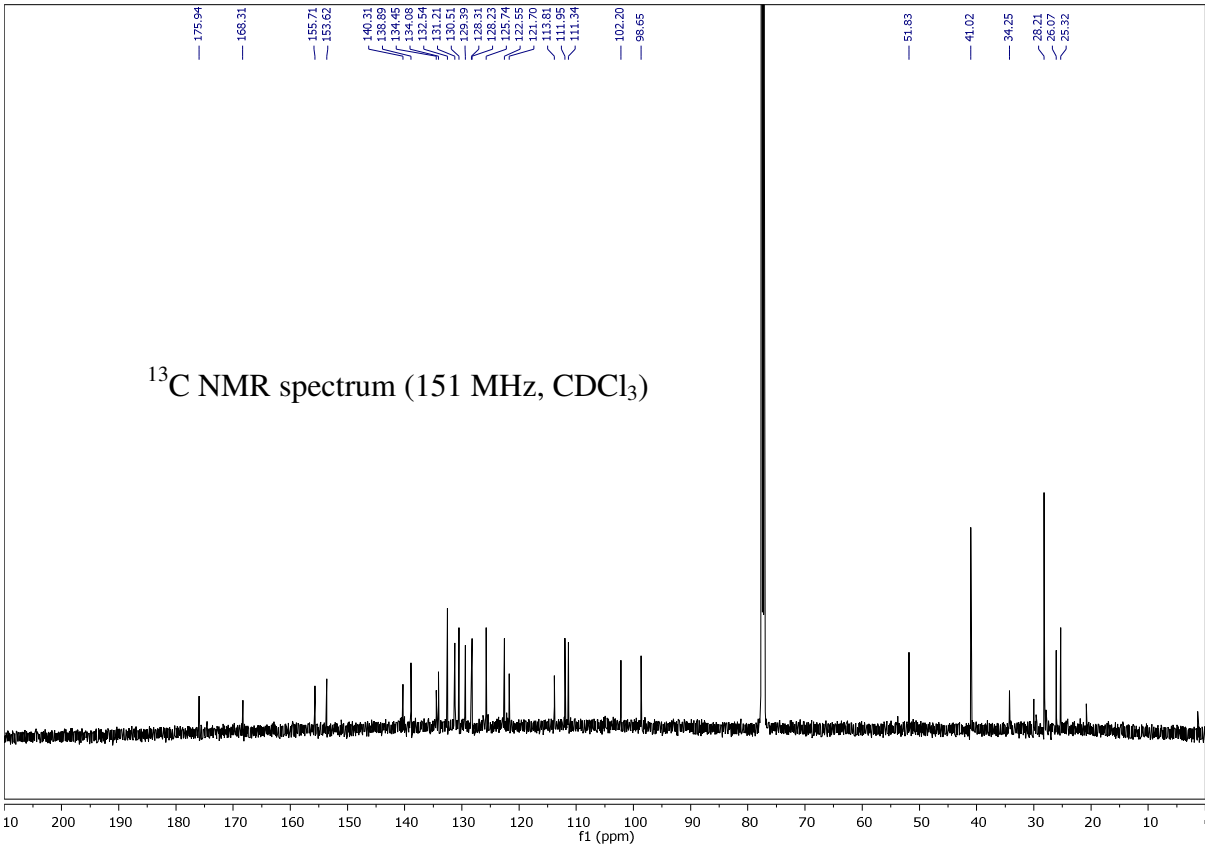
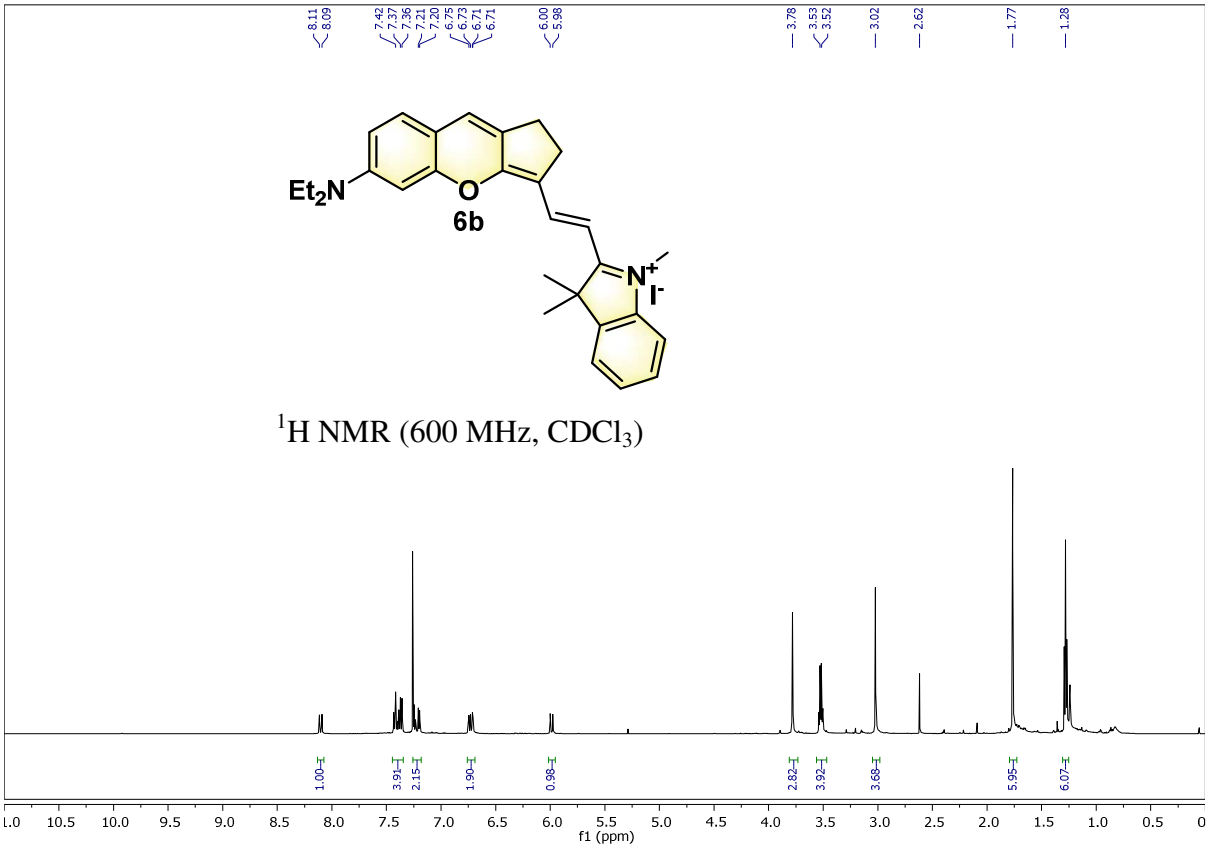


^{13}C NMR spectrum (151 MHz, $CDCl_3$)



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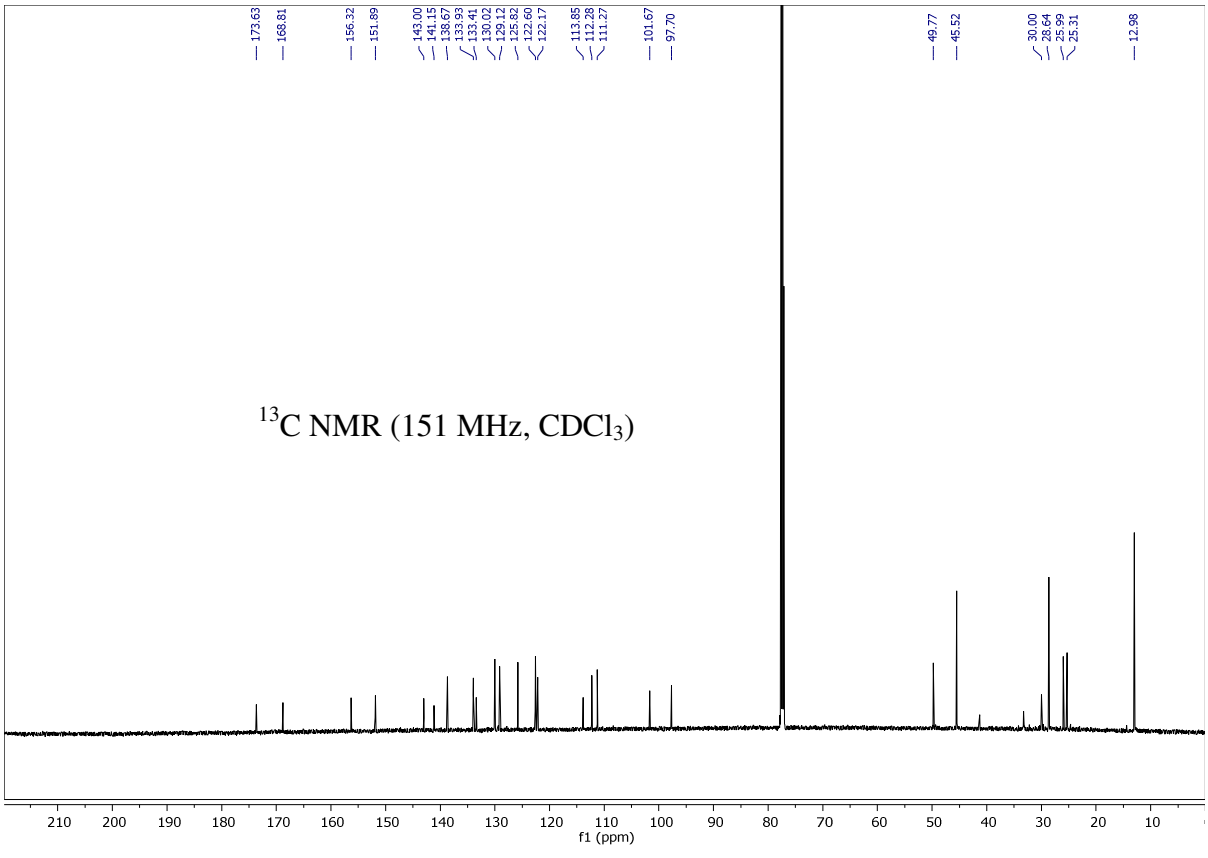

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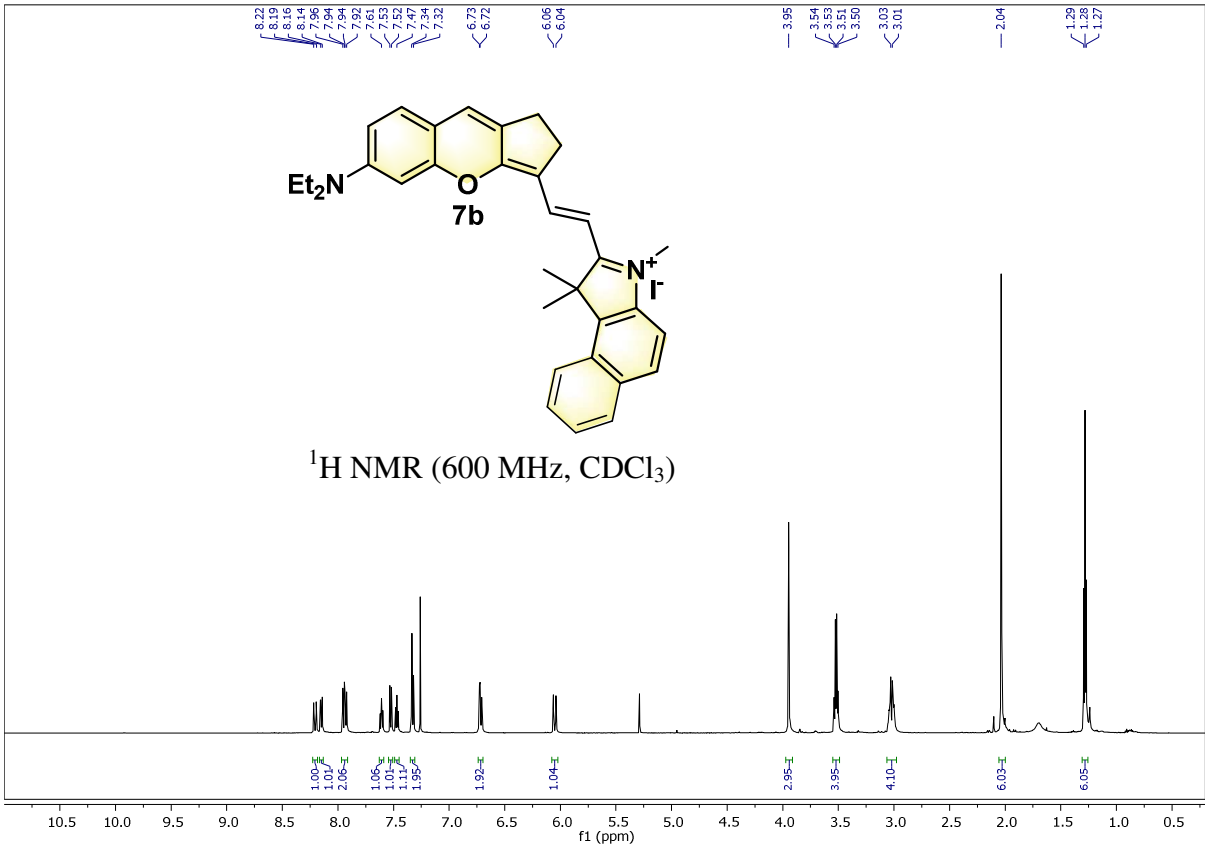
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O
7b
N+
I-
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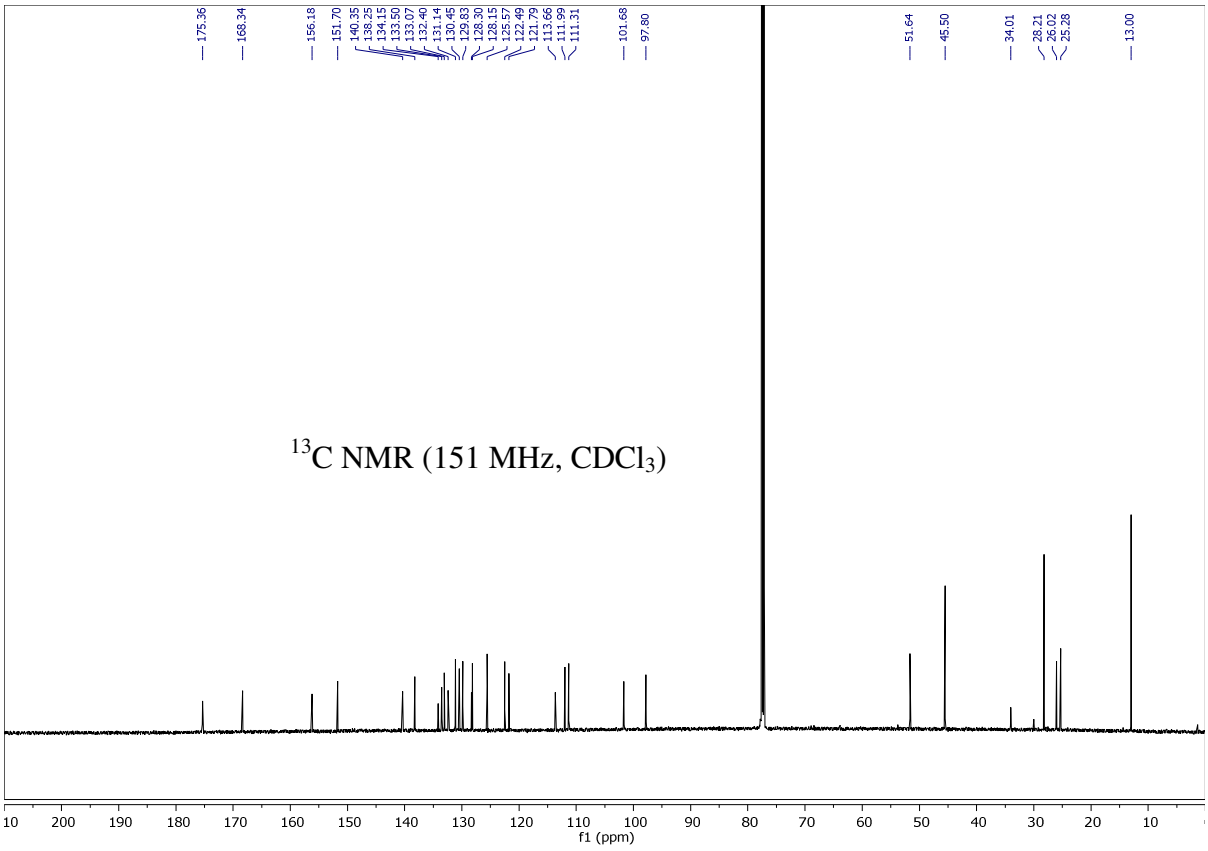
f1 (ppm)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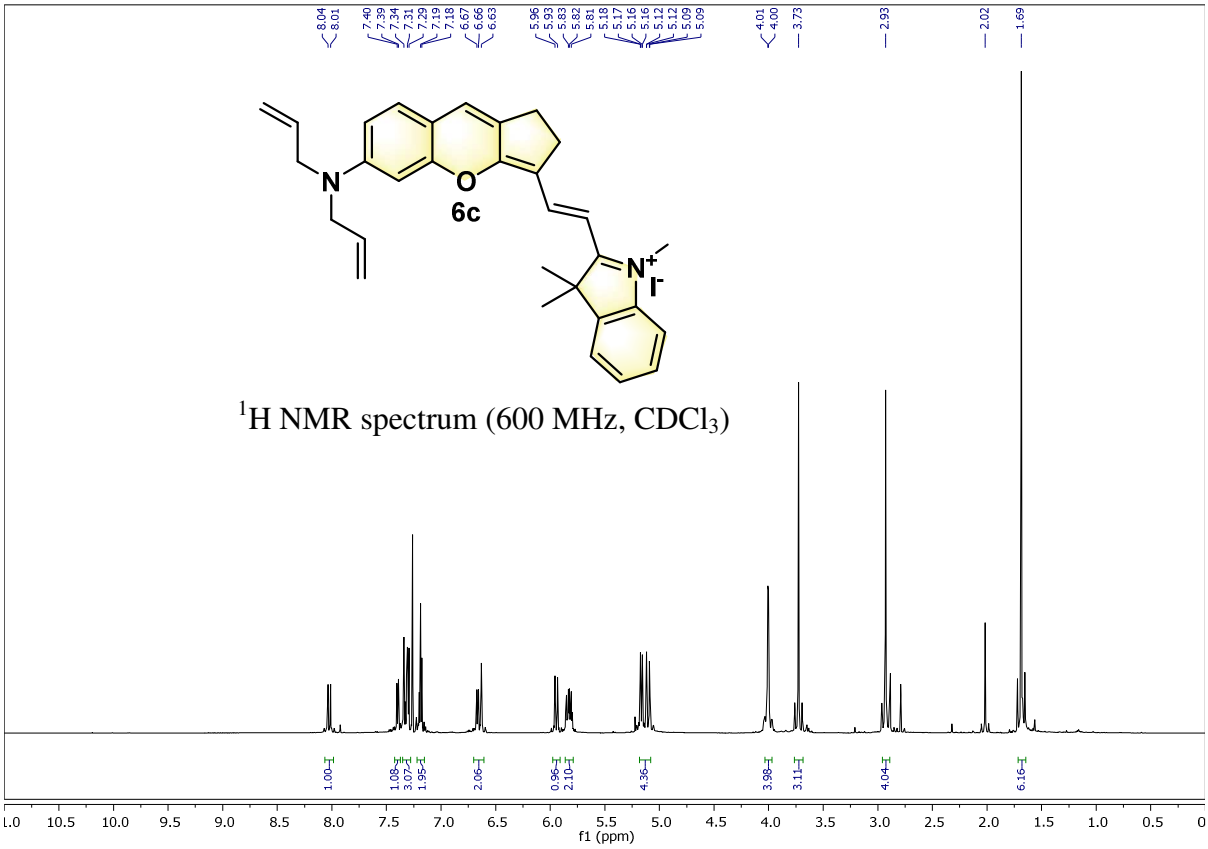
f1 (ppm)



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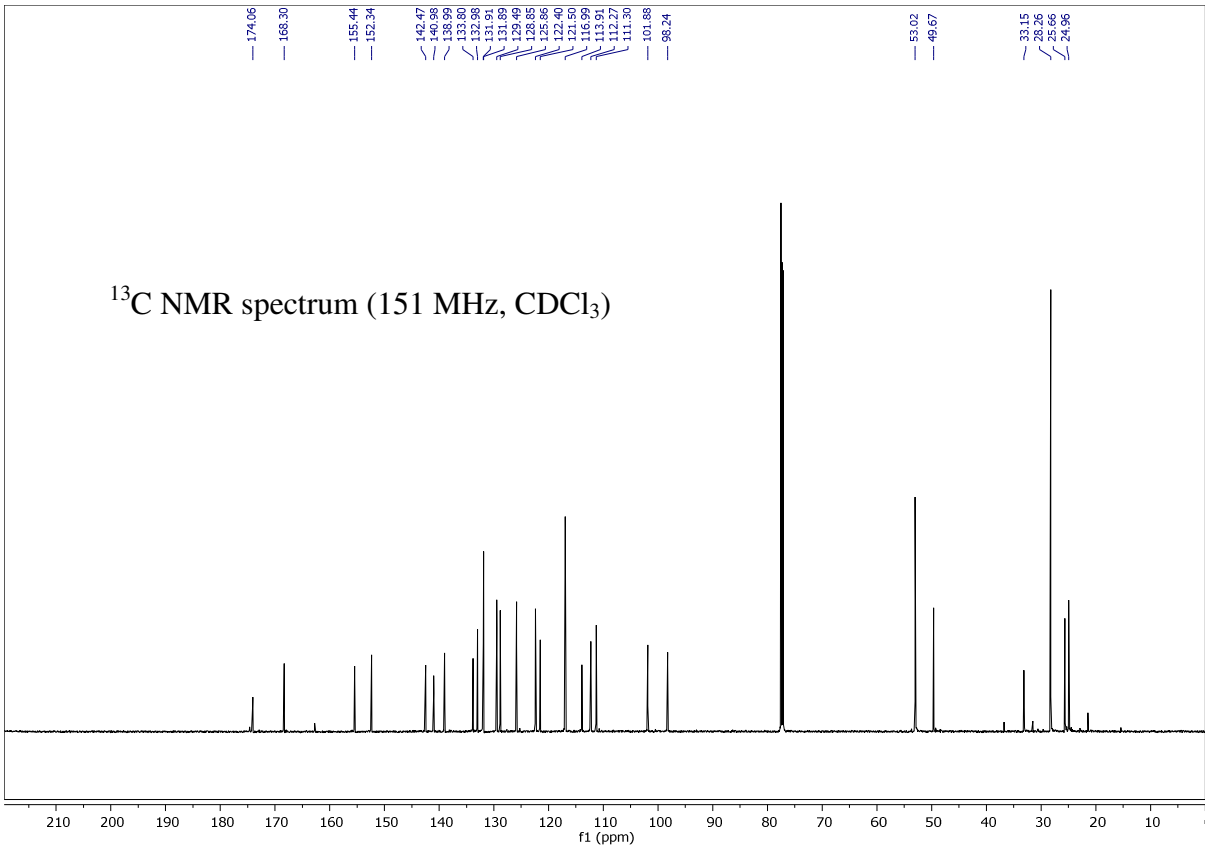
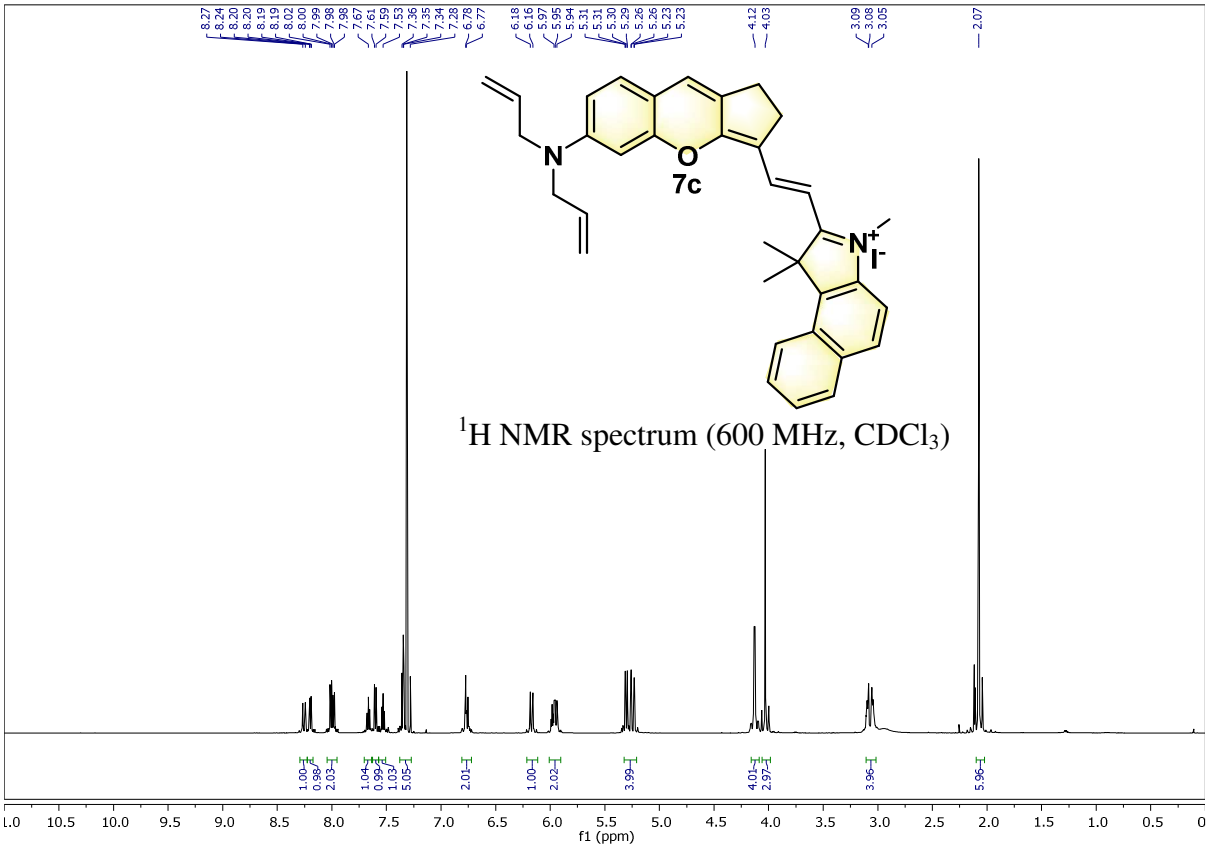


^{13}C NMR spectrum (151 MHz, $CDCl_3$)


7c
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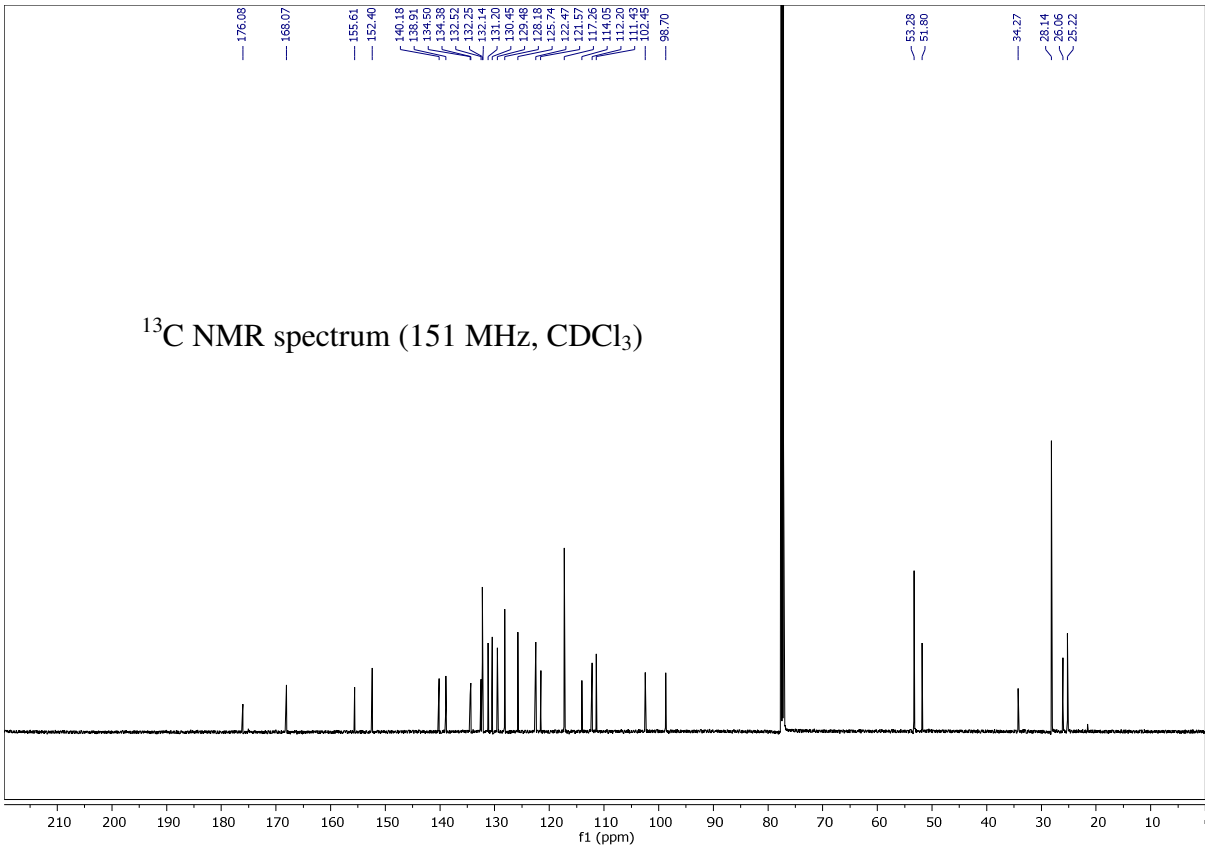
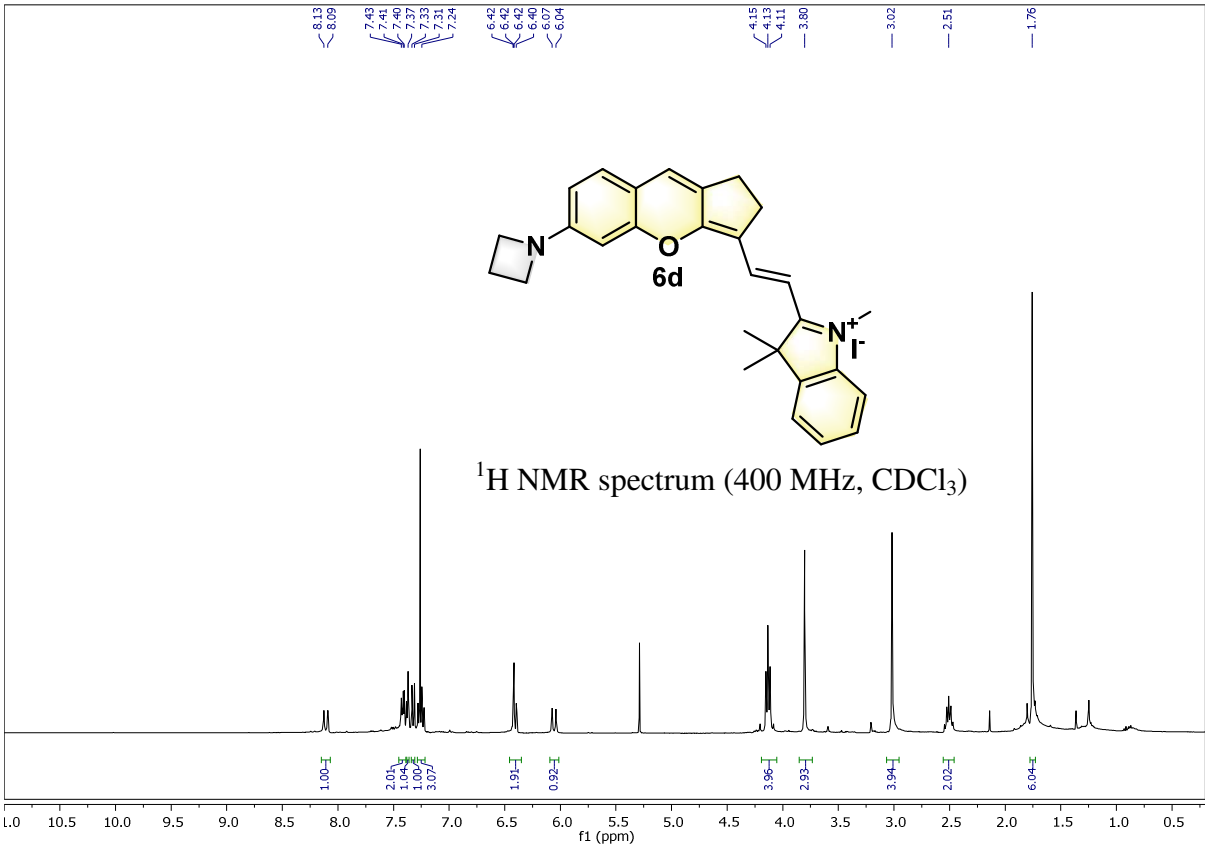
f1 (ppm)


^{13}C NMR spectrum (151 MHz, $CDCl_3$)
f1 (ppm)



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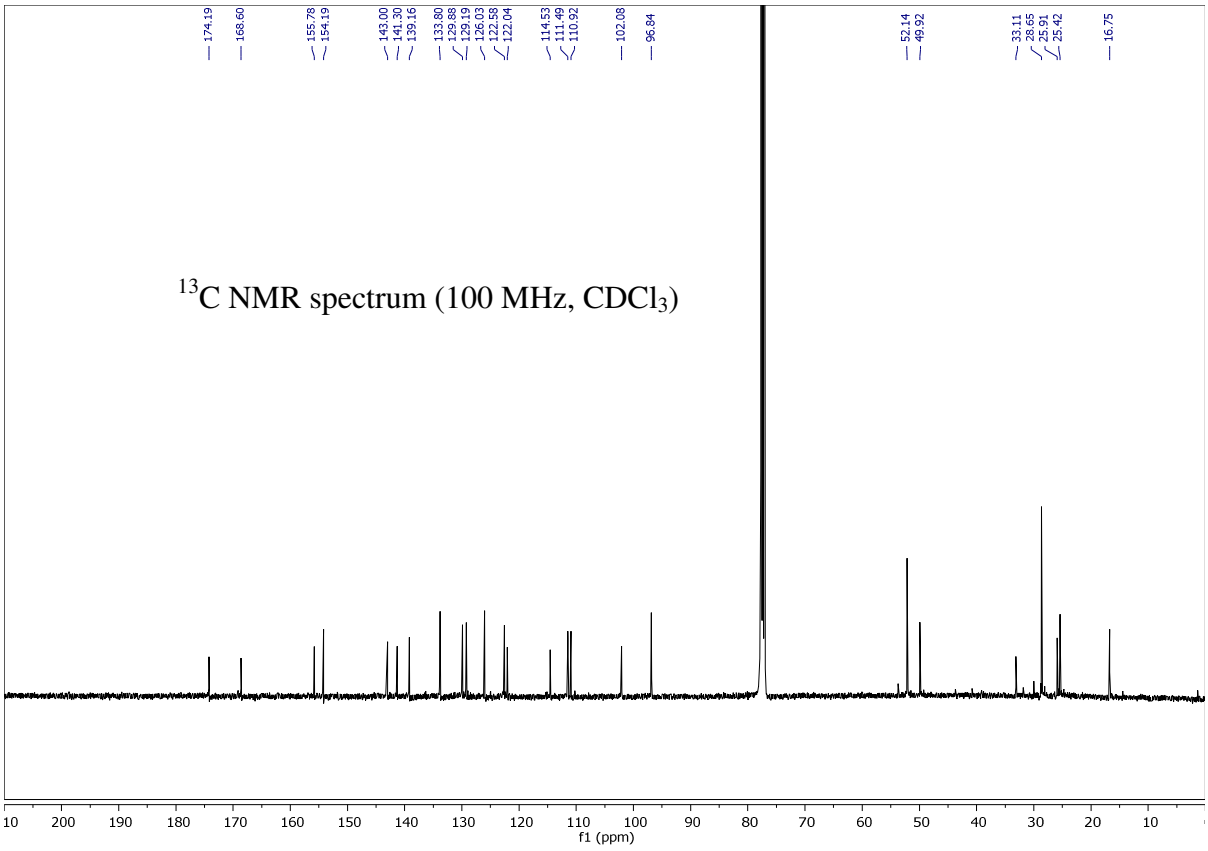

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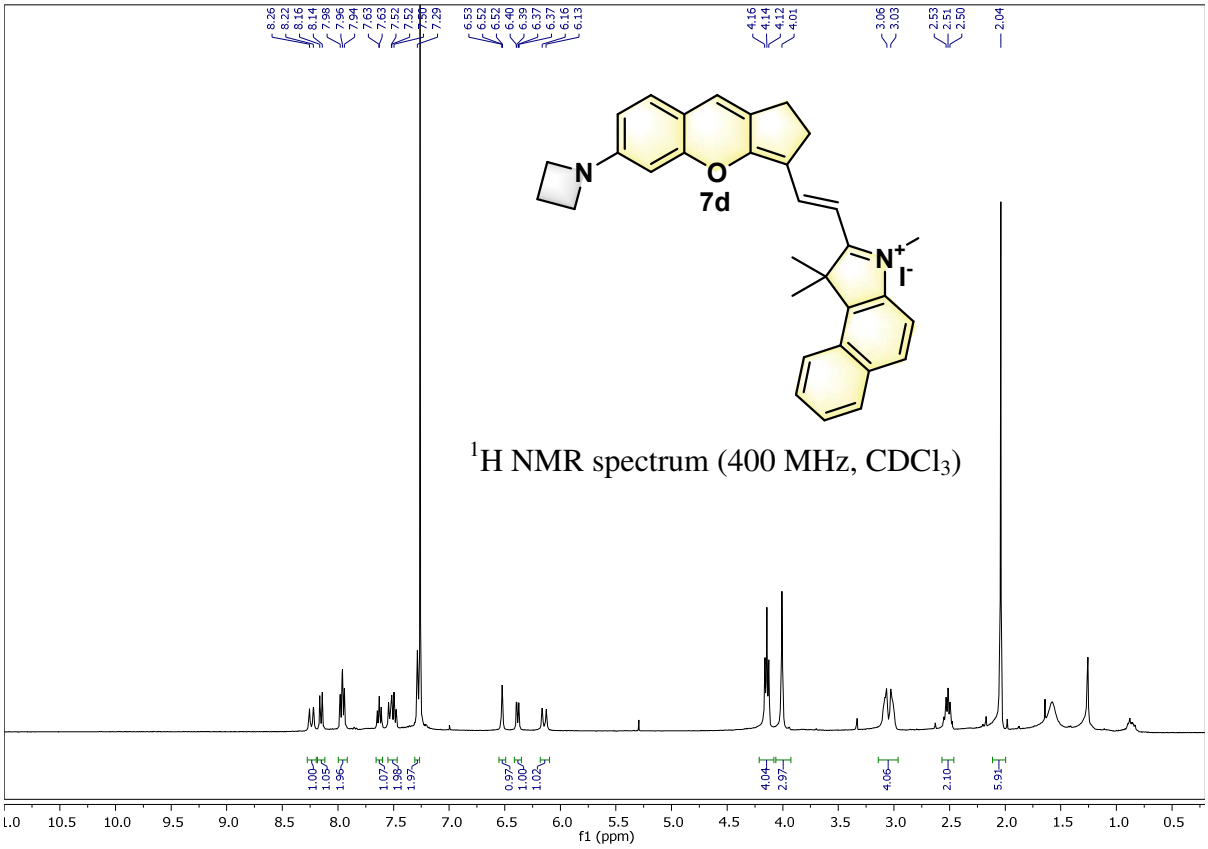
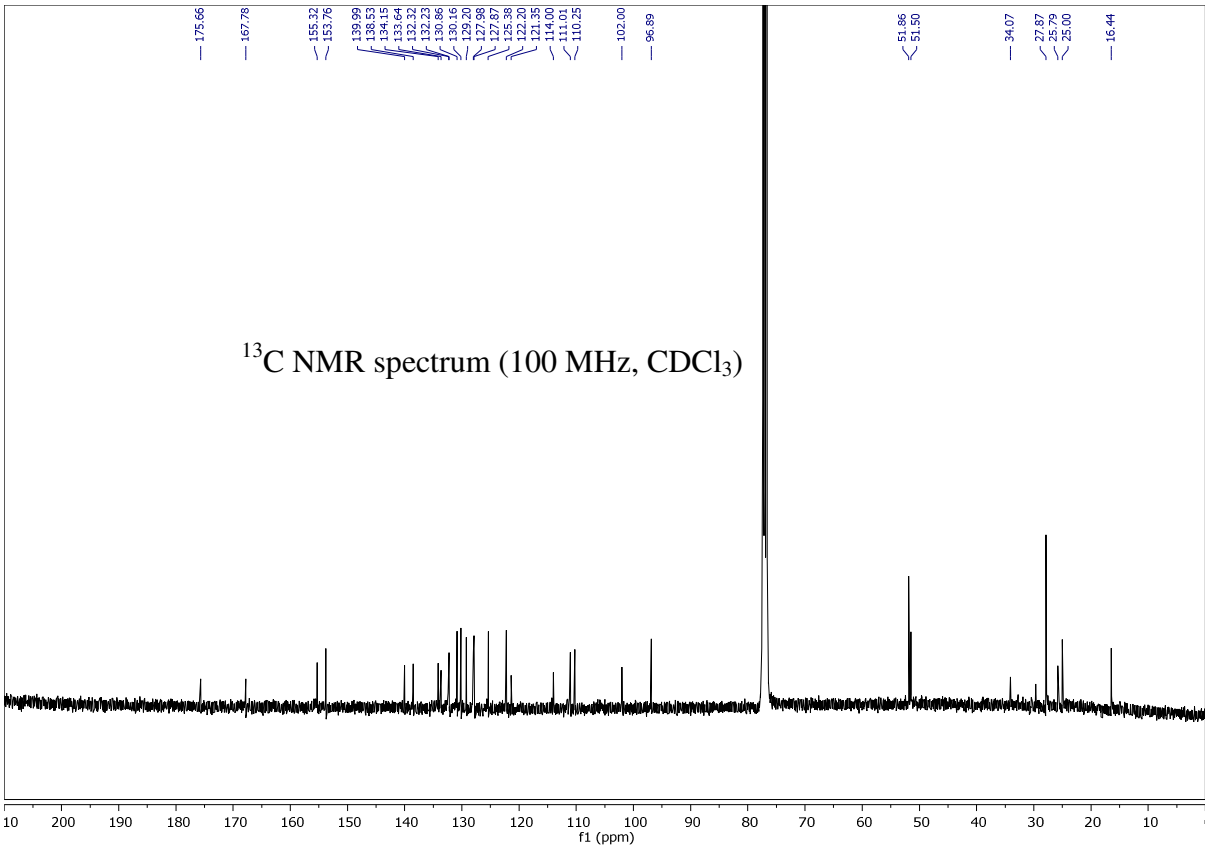
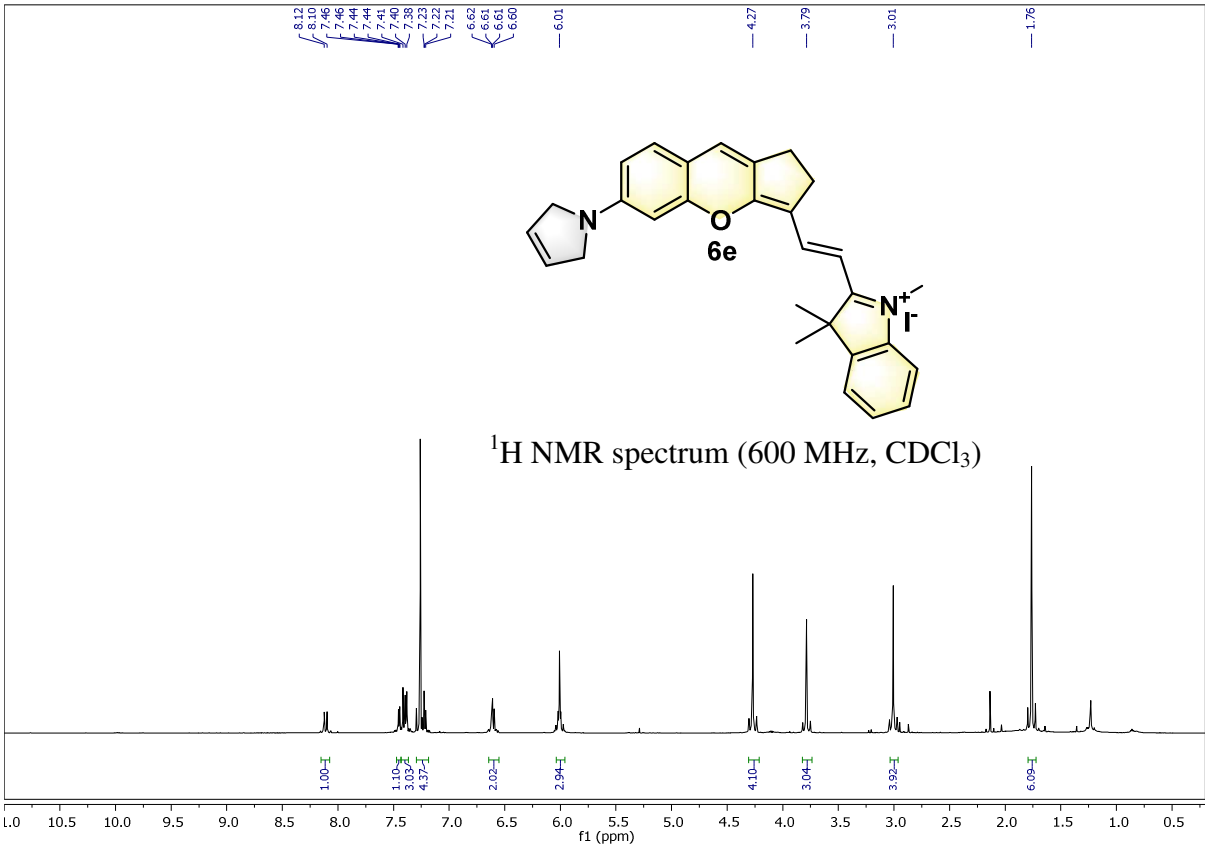
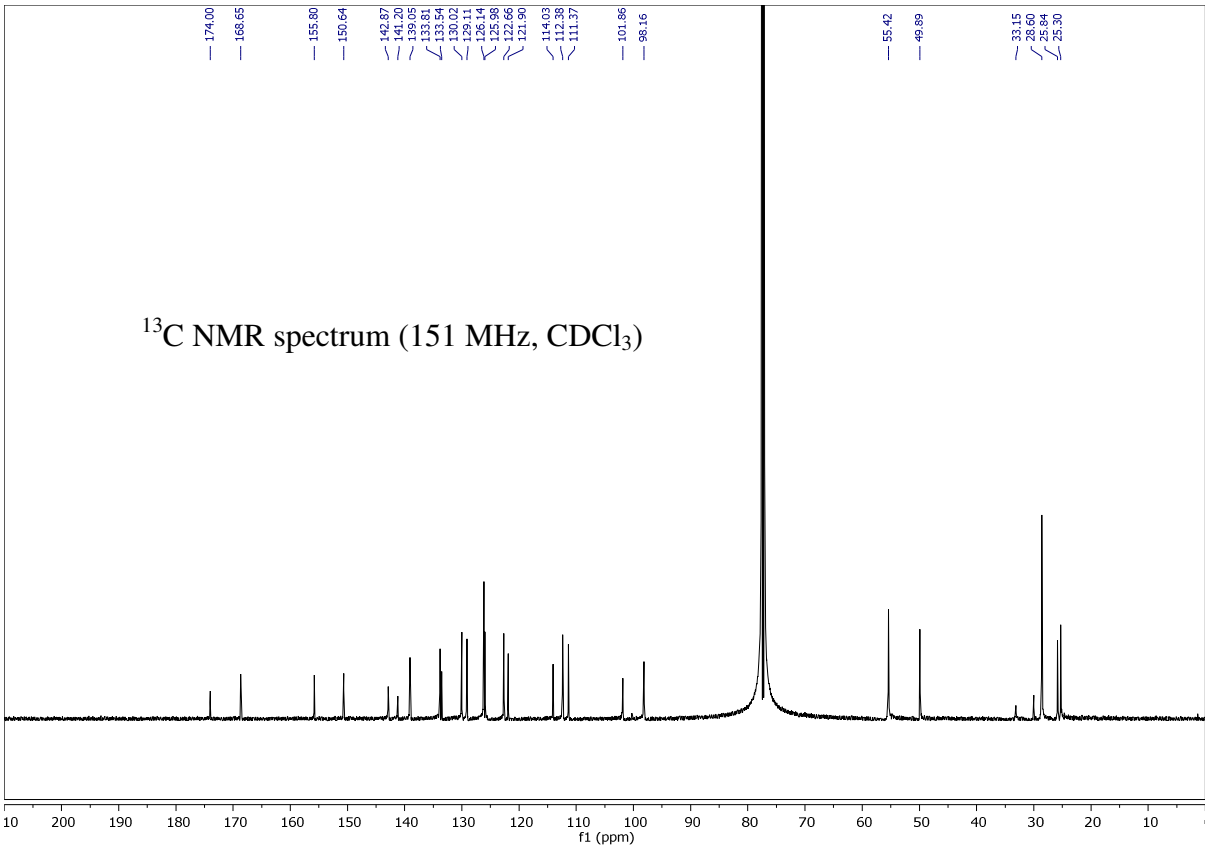

7d
^{1}H NMR spectrum (400 MHz, $CDCl_3$)
f1 (ppm)

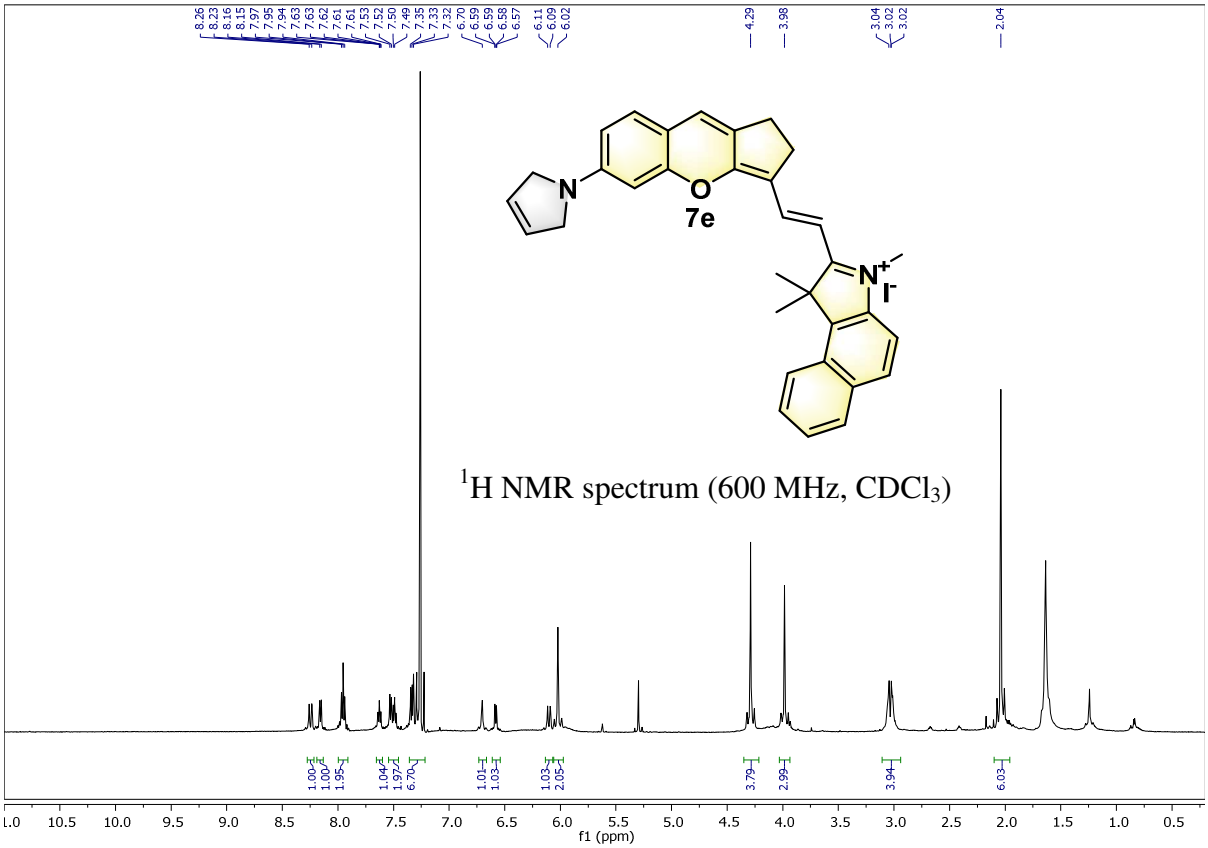

^{13}C NMR spectrum (100 MHz, $CDCl_3$)
f1 (ppm)

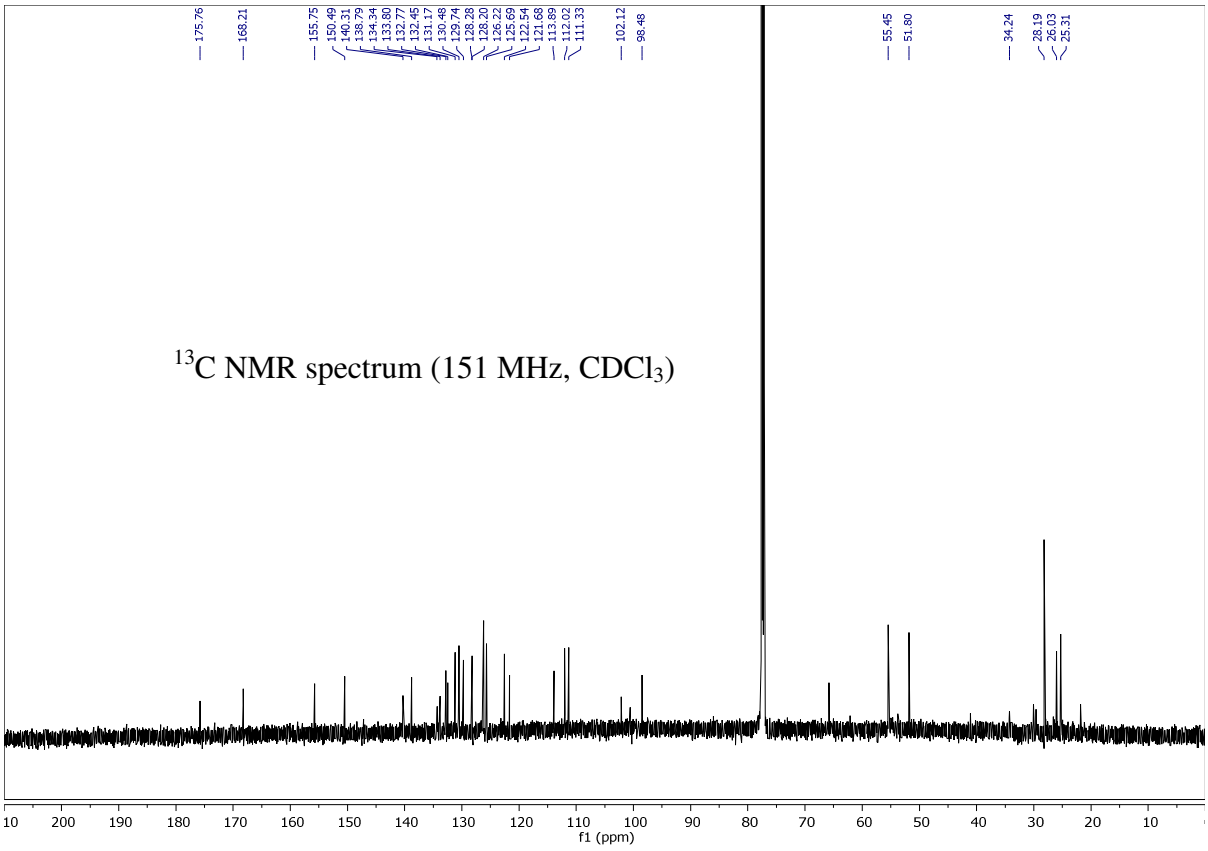


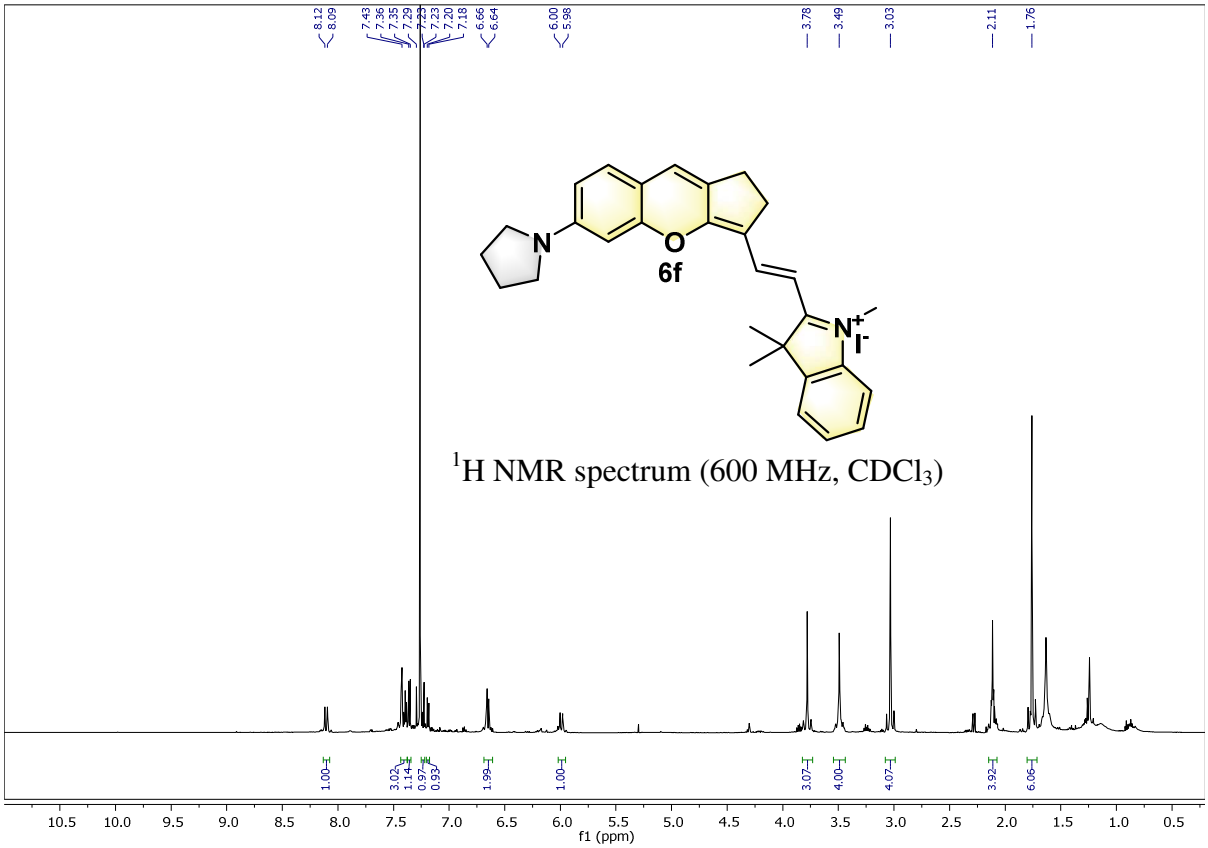
^{1}H NMR spectrum (600 MHz, $CDCl_3$)



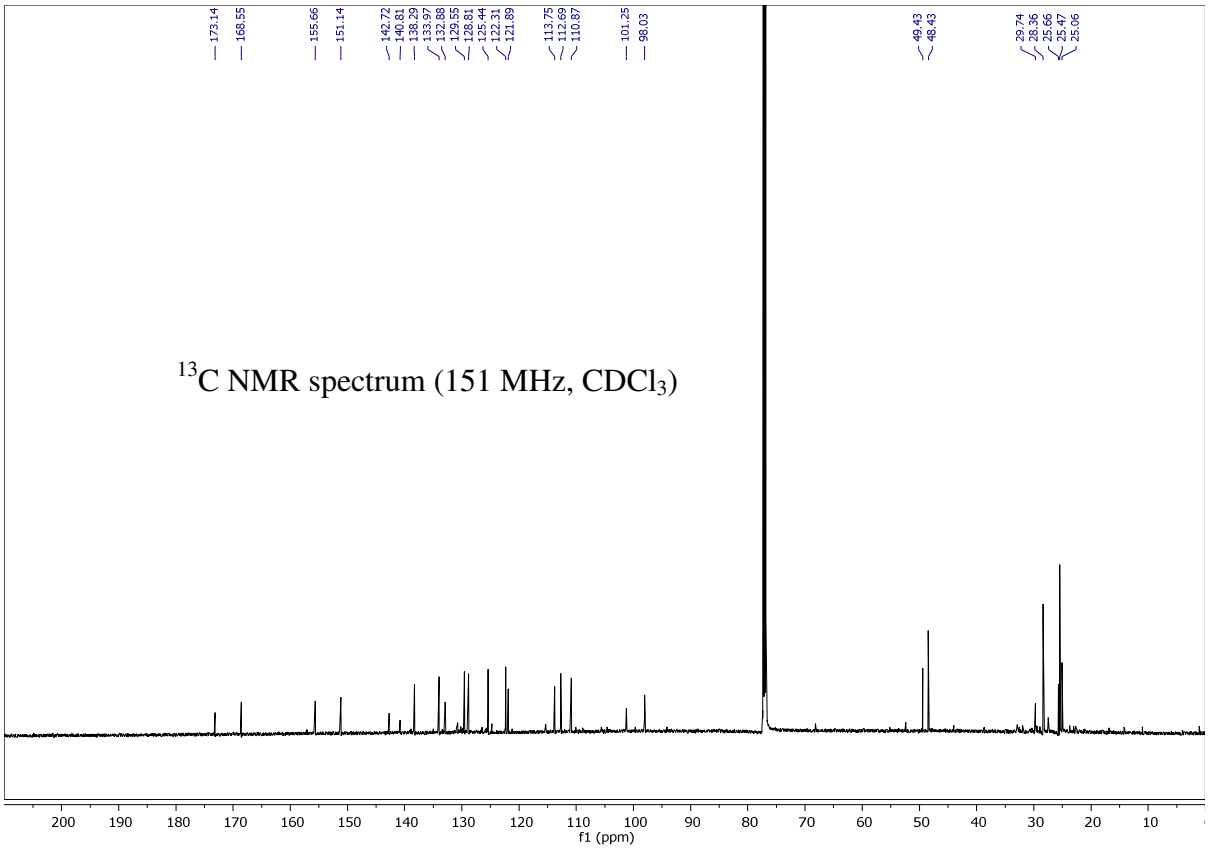
^{13}C NMR spectrum (151 MHz, $CDCl_3$)


7e
^{1}H NMR spectrum (600 MHz, $CDCl_3$)
f1 (ppm)

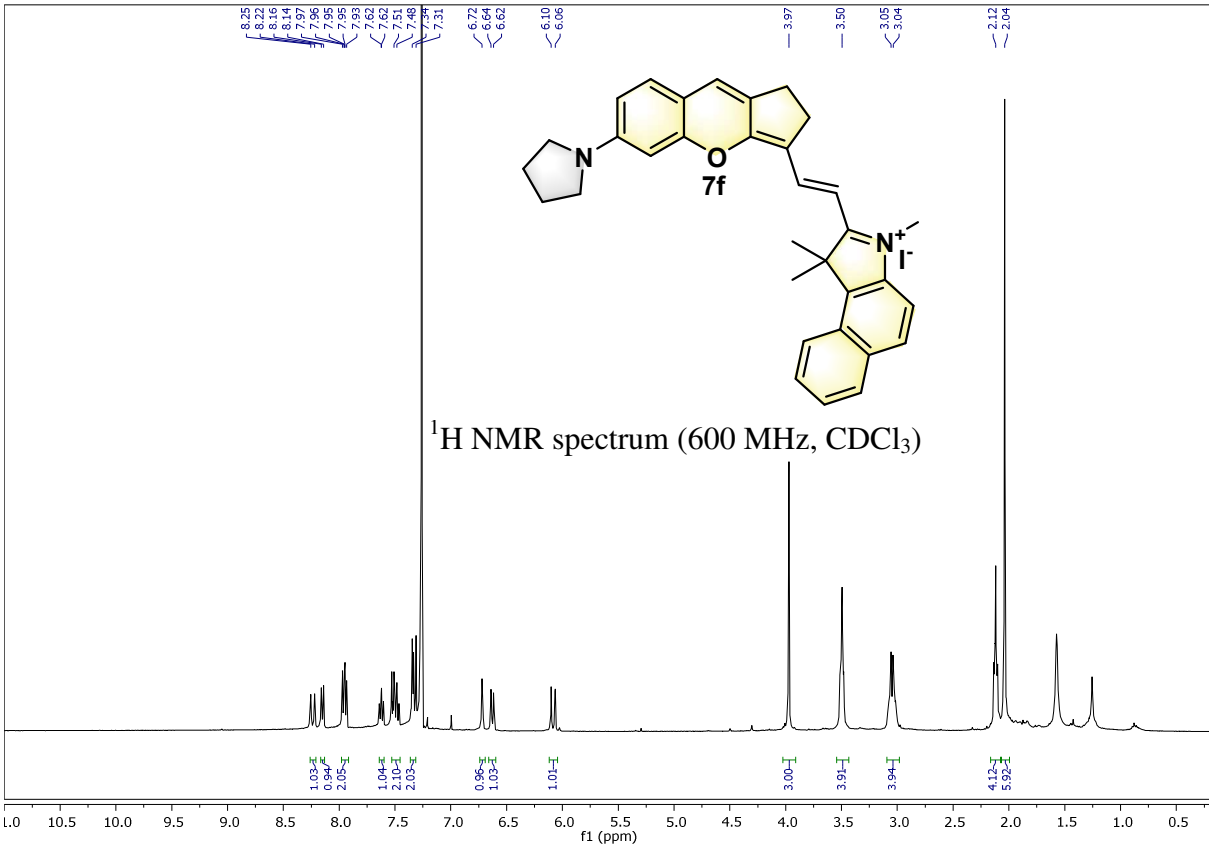

^{13}C NMR spectrum (151 MHz, $CDCl_3$)
f1 (ppm)

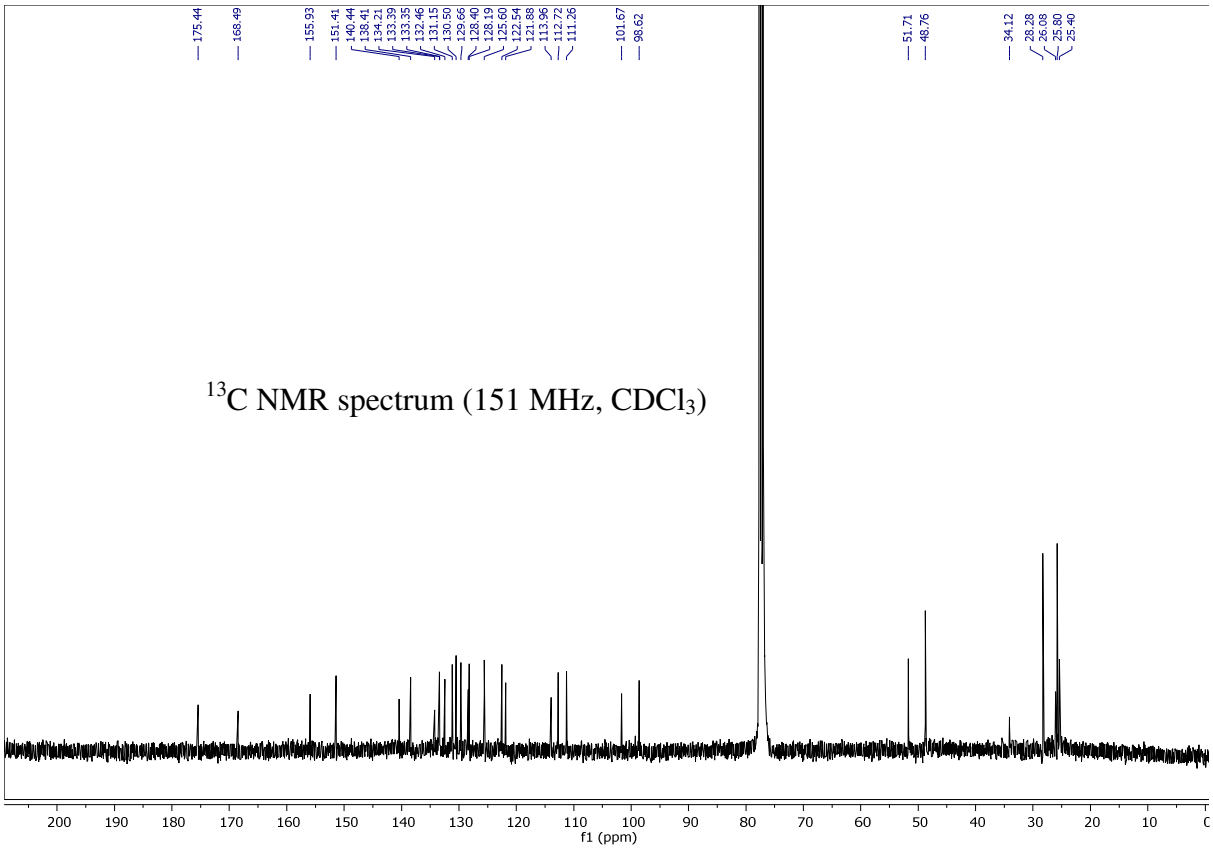


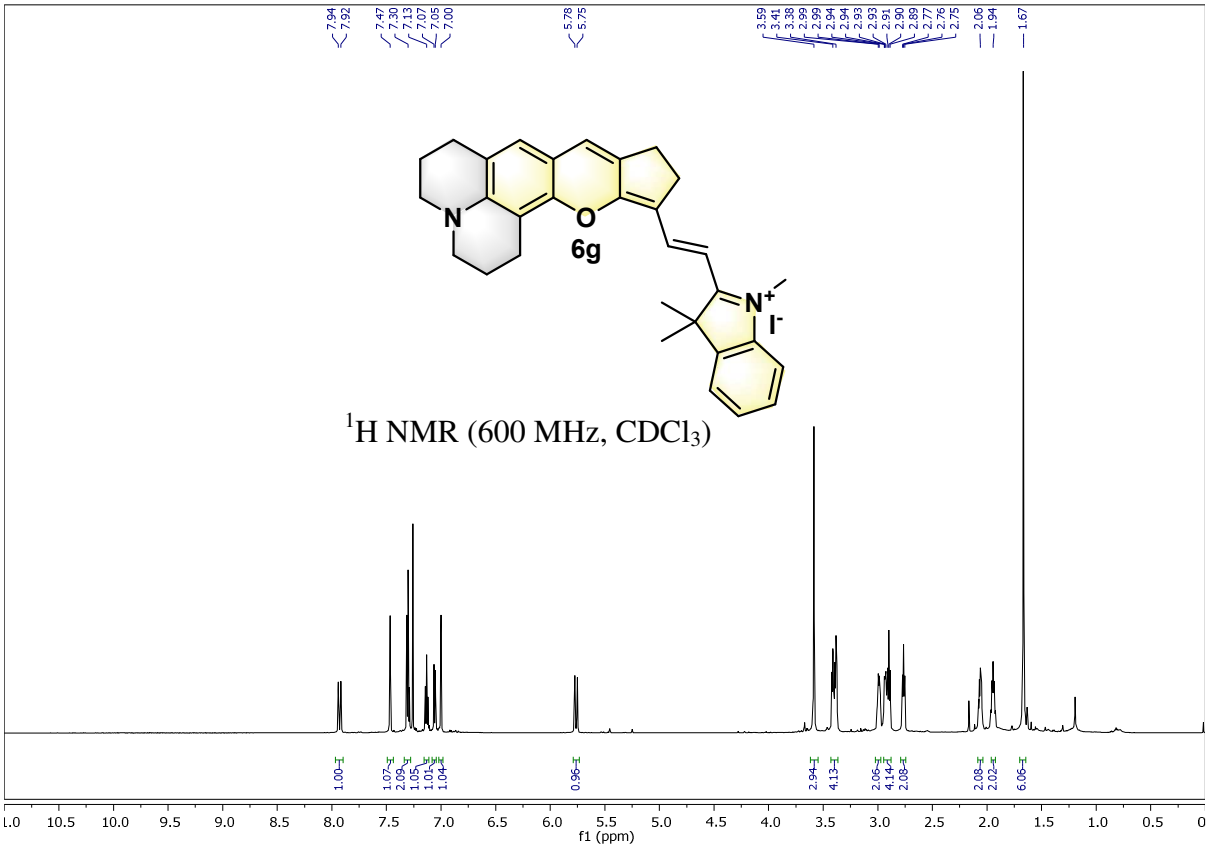
^{1}H NMR spectrum (600 MHz, $CDCl_3$)

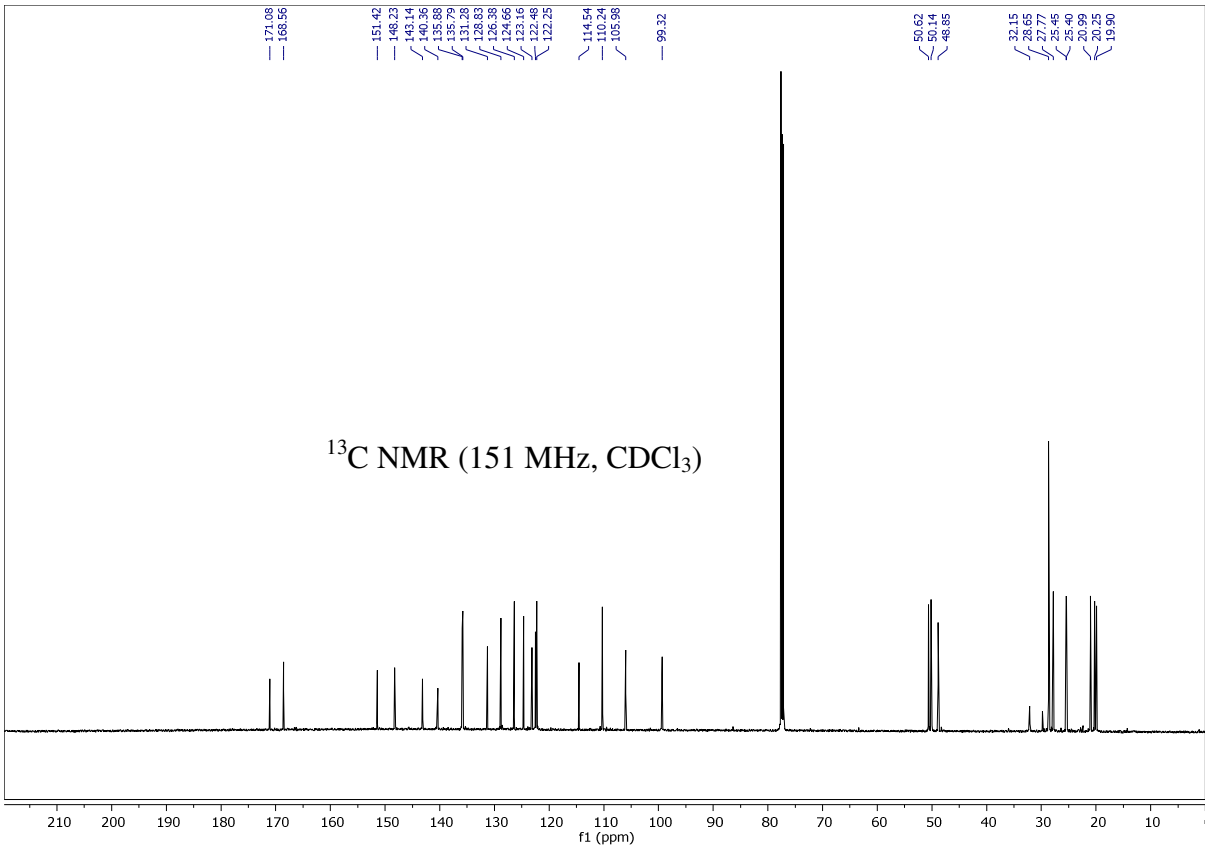


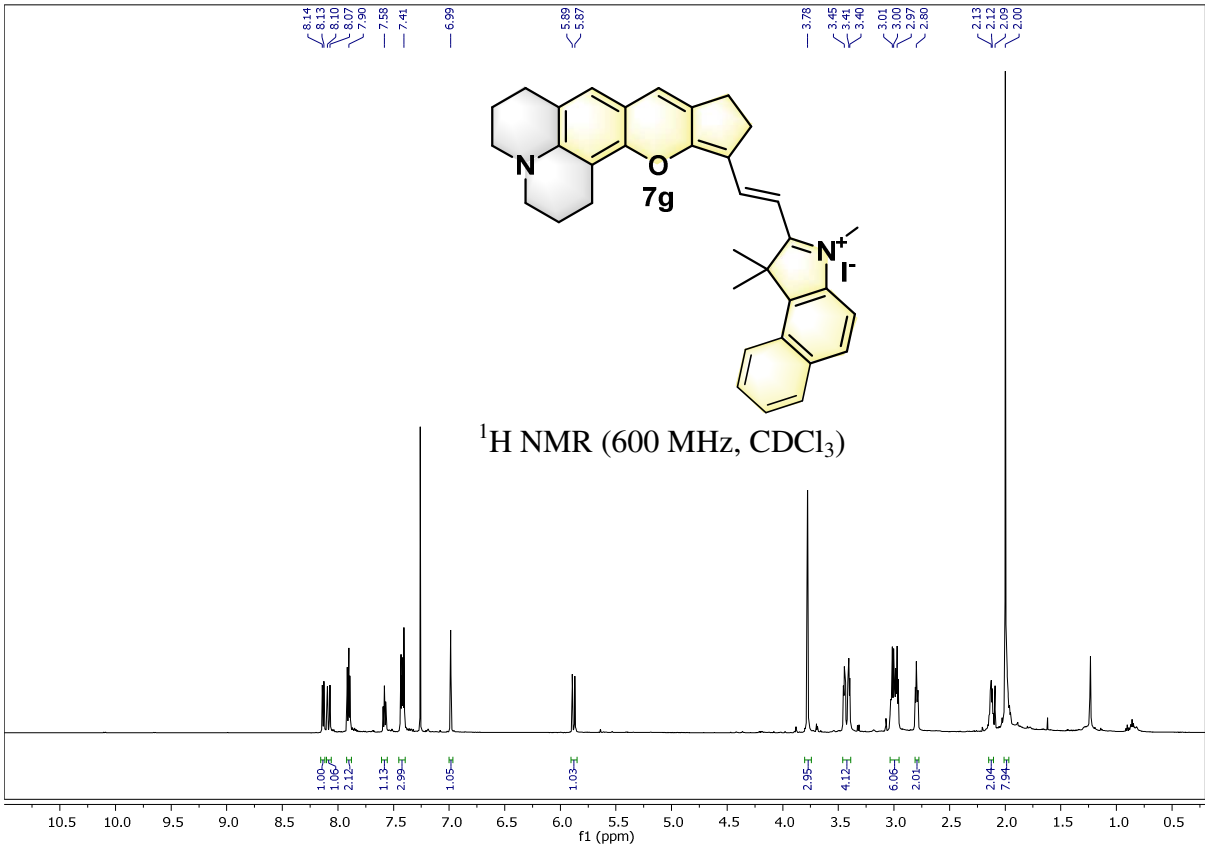
^{13}C NMR spectrum (151 MHz, $CDCl_3$)

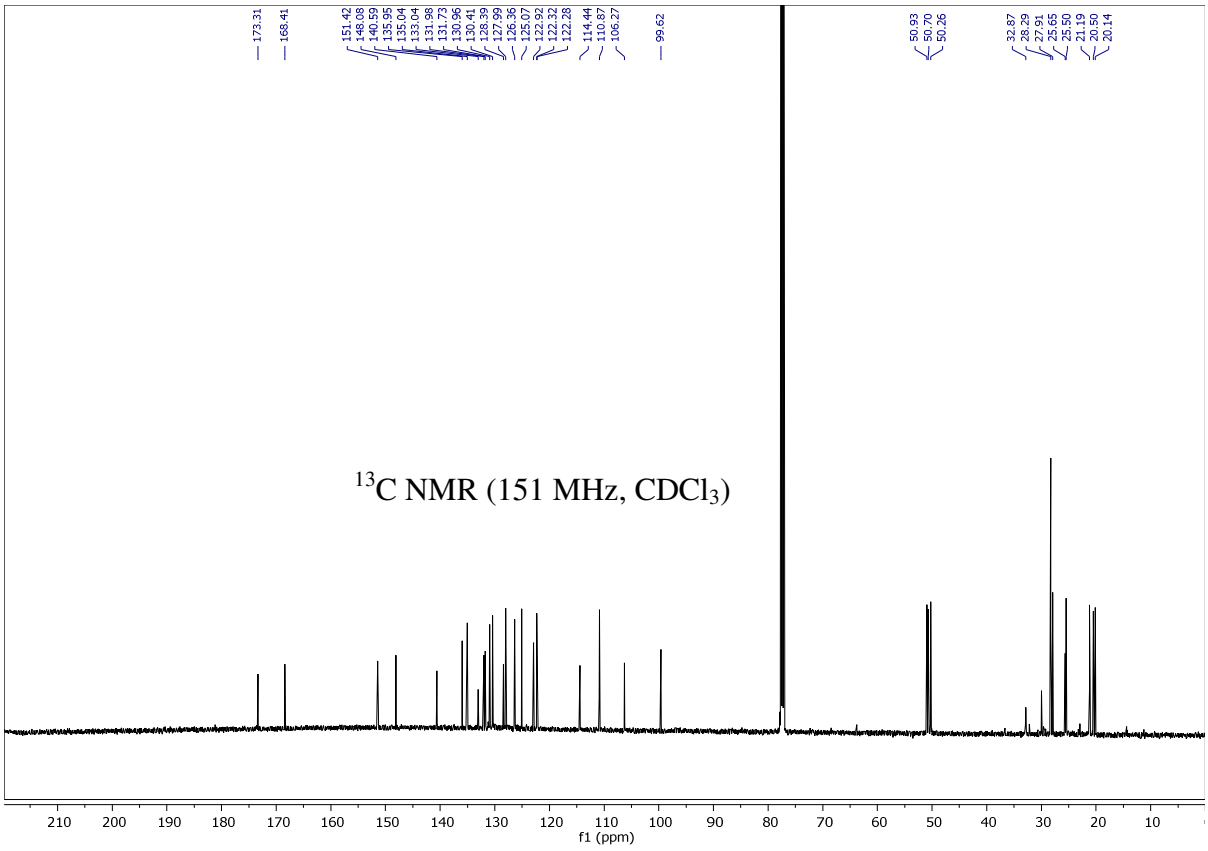

7f
^{1}H NMR spectrum (600 MHz, $CDCl_3$)
f1 (ppm)

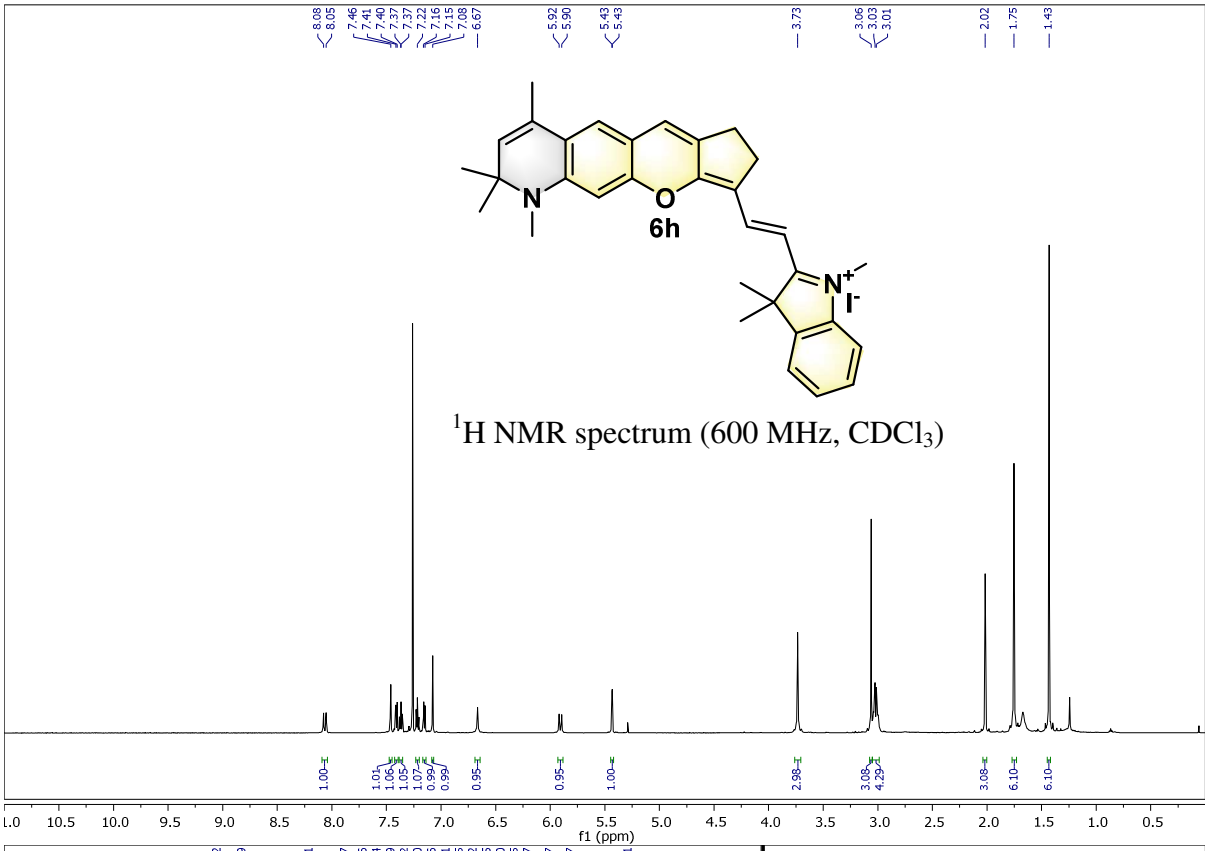

^{13}C NMR spectrum (151 MHz, $CDCl_3$)
f1 (ppm)

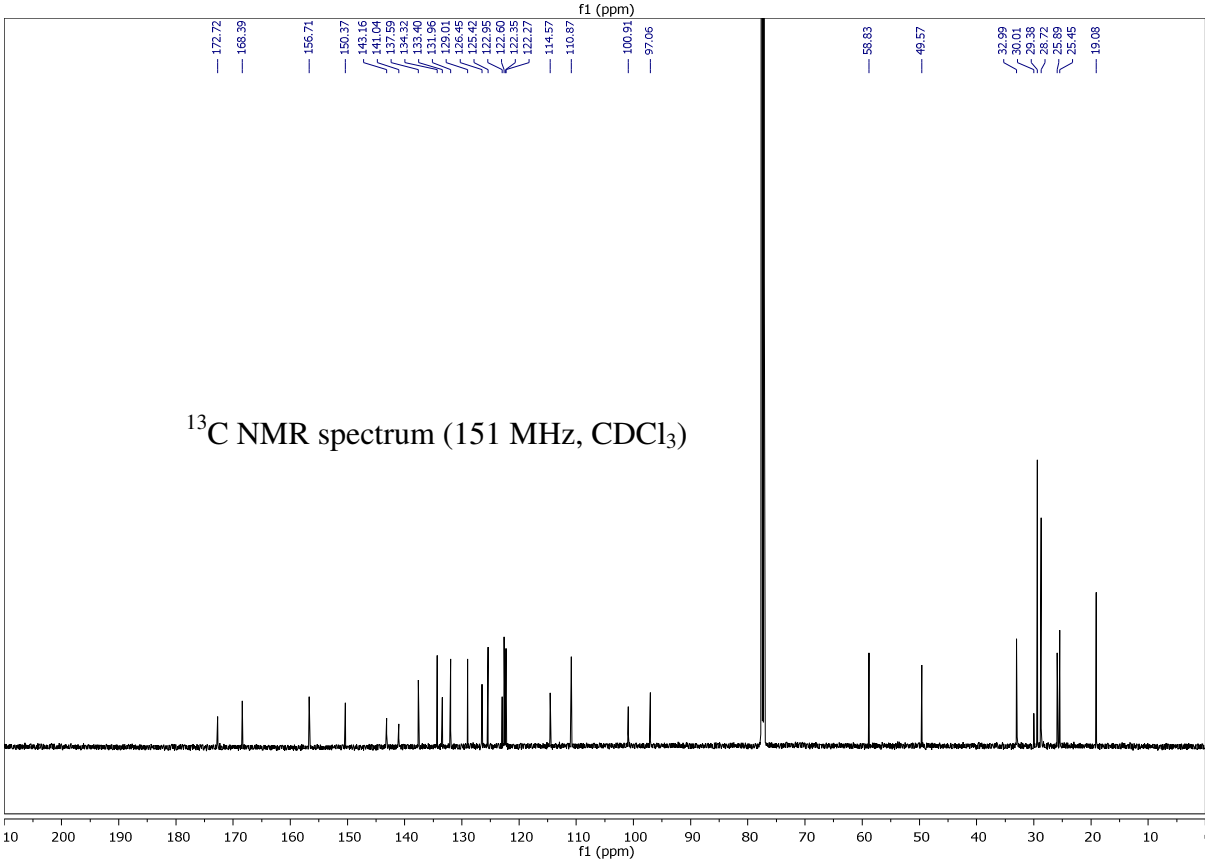

6g
^{1}H NMR (600 MHz, CDCl$_3$)
f1 (ppm)

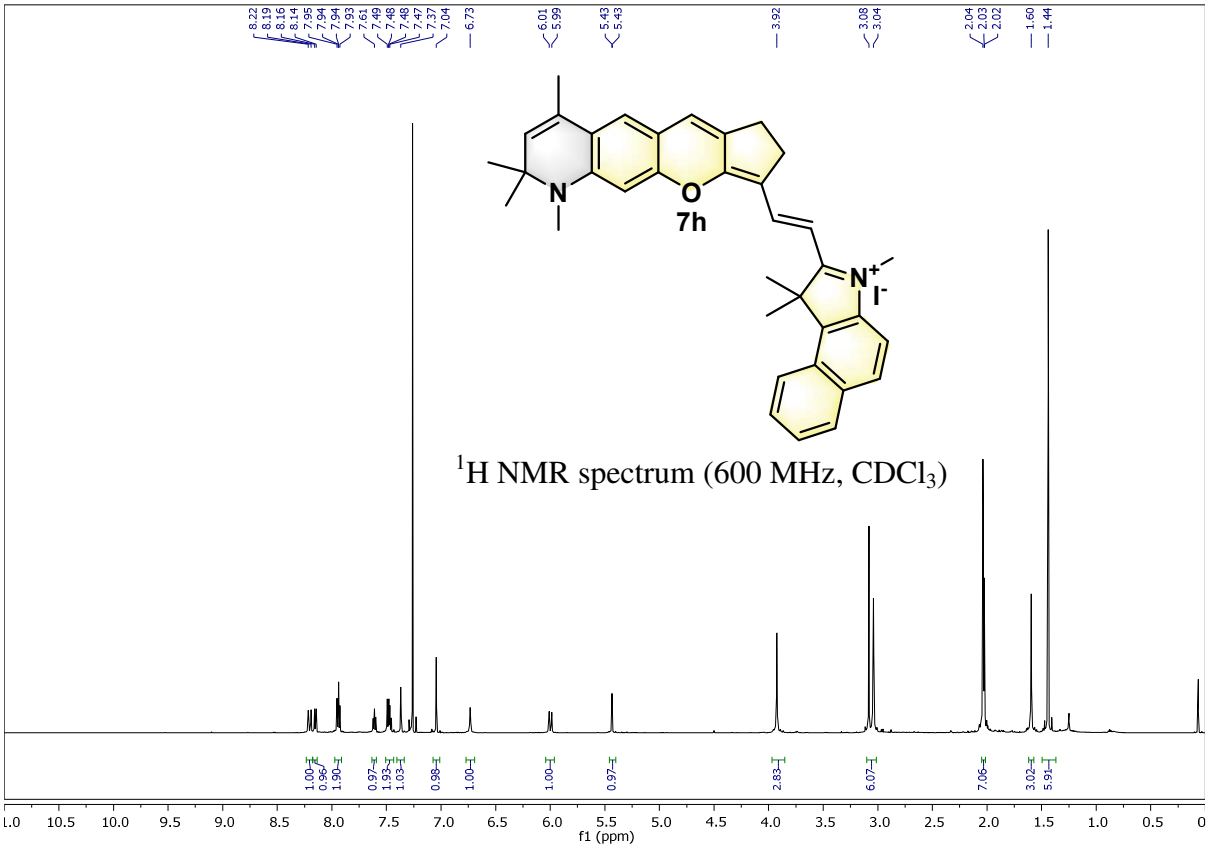

^{13}C NMR (151 MHz, CDCl$_3$)
f1 (ppm)


8.14
8.13
8.10
8.07
7.90
7.58
7.41
6.99
5.89
5.87
3.78
3.45
3.41
3.40
3.01
3.00
2.97
2.80
2.13
2.12
2.09
2.00
N
O
7g
N+
I-
1H NMR (600 MHz, CDCl3)
1.00
1.06
2.12
1.13
2.99
1.05
1.03
2.95
4.12
6.06
2.01
2.04
7.94
10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
f1 (ppm)

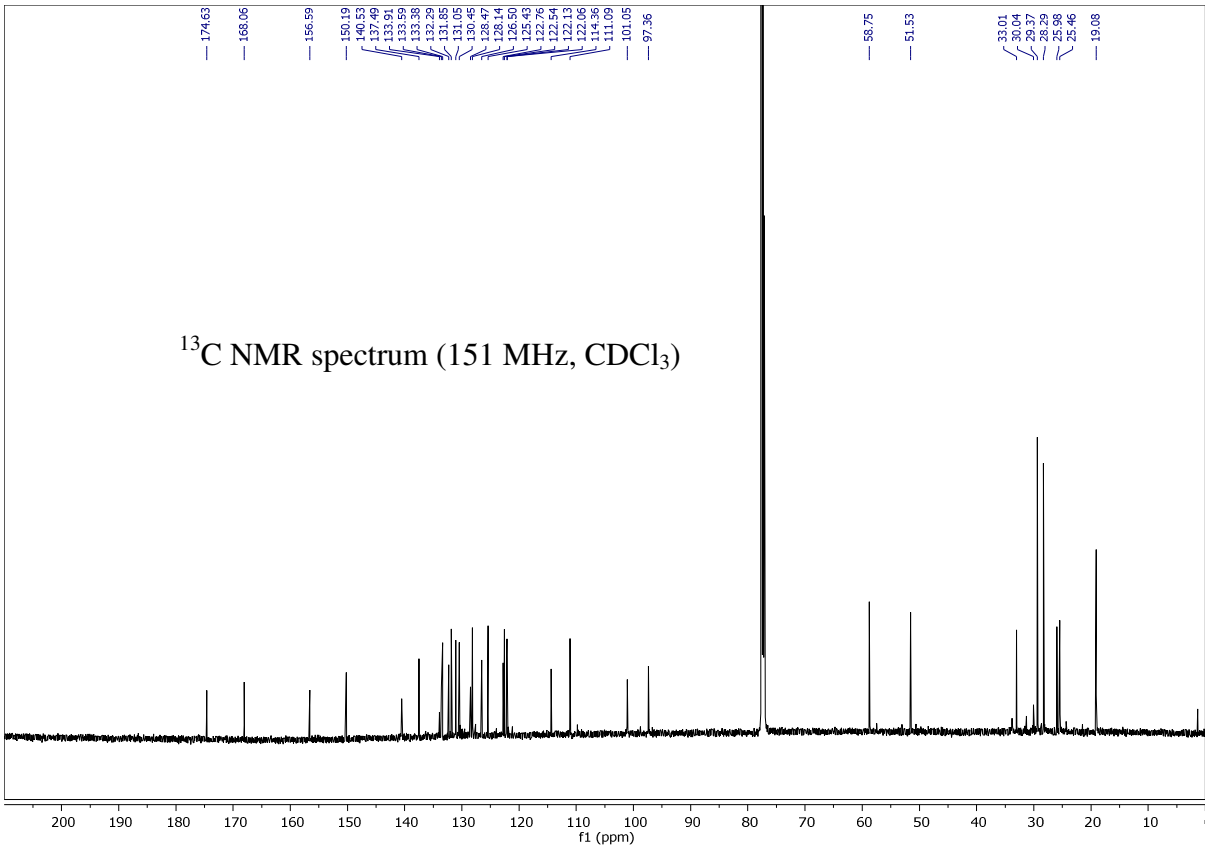

173.31
168.41
151.42
148.08
140.59
135.95
135.04
133.04
131.98
131.73
130.96
130.41
128.39
127.99
126.36
125.07
122.92
122.32
122.28
114.44
110.87
106.27
99.62
50.93
50.70
50.26
32.87
28.29
27.91
25.65
25.50
21.19
20.50
20.14
13C NMR (151 MHz, CDCl3)
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10
f1 (ppm)


6h
1H NMR spectrum (600 MHz, CDCl3)
f1 (ppm)


13C NMR spectrum (151 MHz, CDCl3)
f1 (ppm)



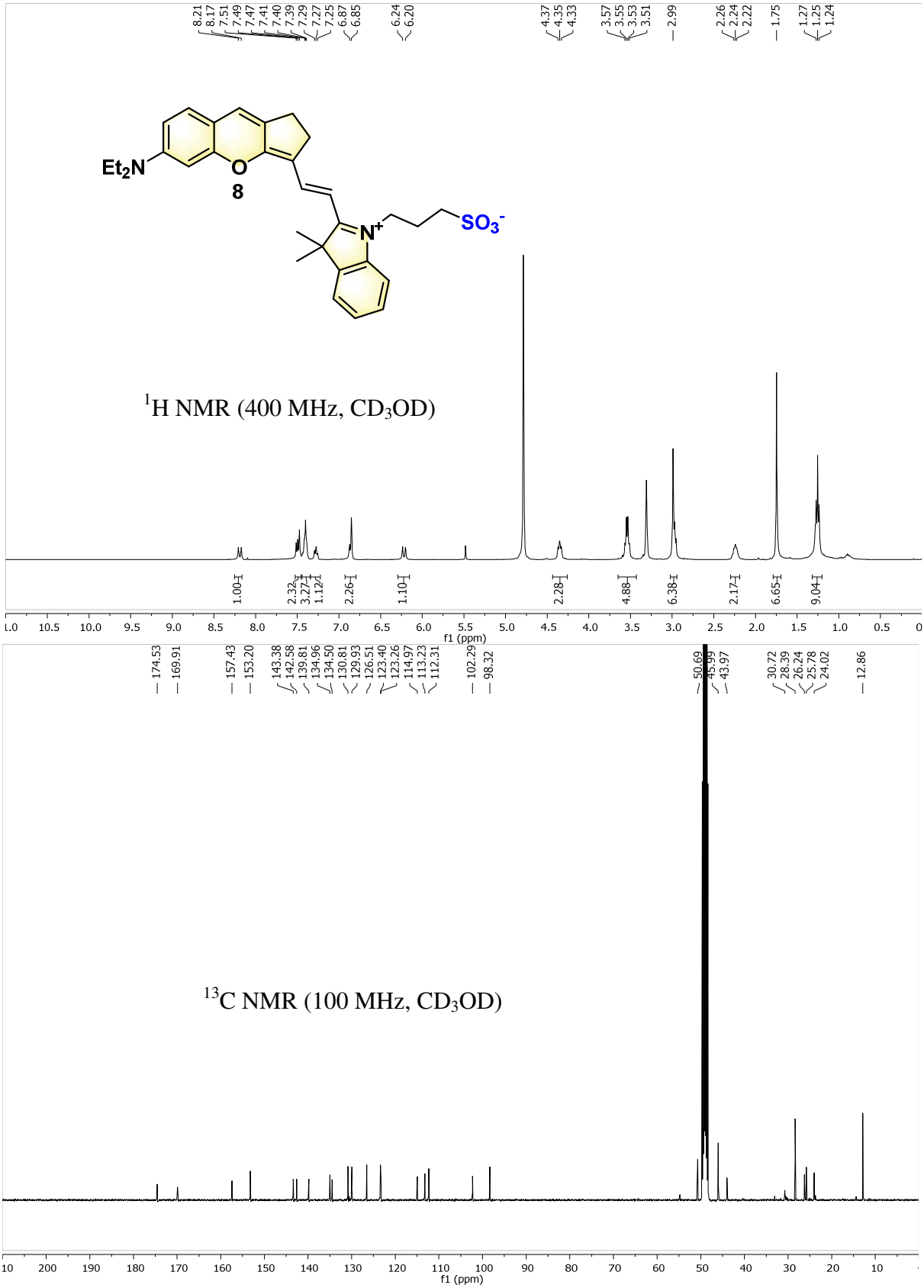
^{1}H NMR spectrum (600 MHz, $CDCl_3$)



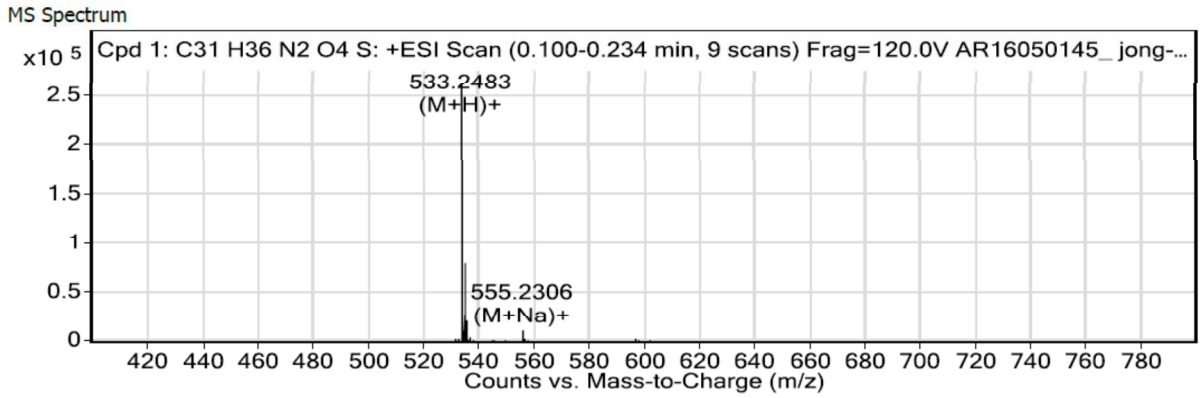
^{13}C NMR spectrum (151 MHz, $CDCl_3$)

2) NMR, ESI-MS (LR and HR) spectra and RP-HPLC elution profiles of water-soluble *nor*-DHX-hemicyanine fused NIR dyes

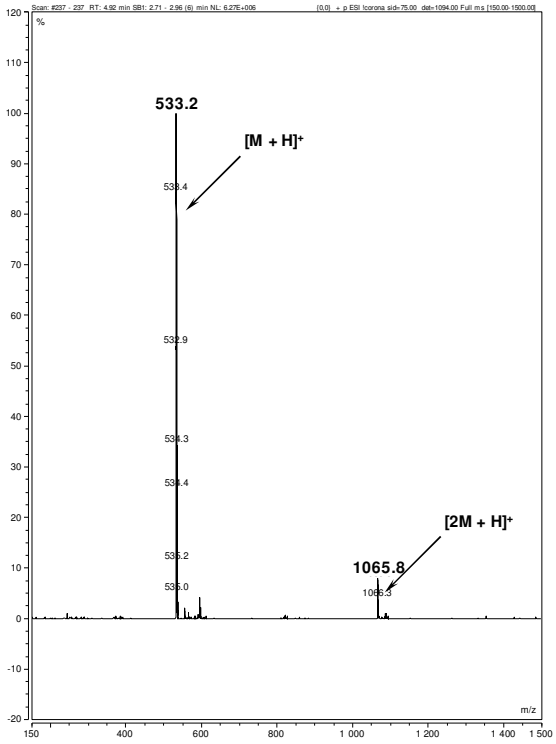
Fluorophore 8



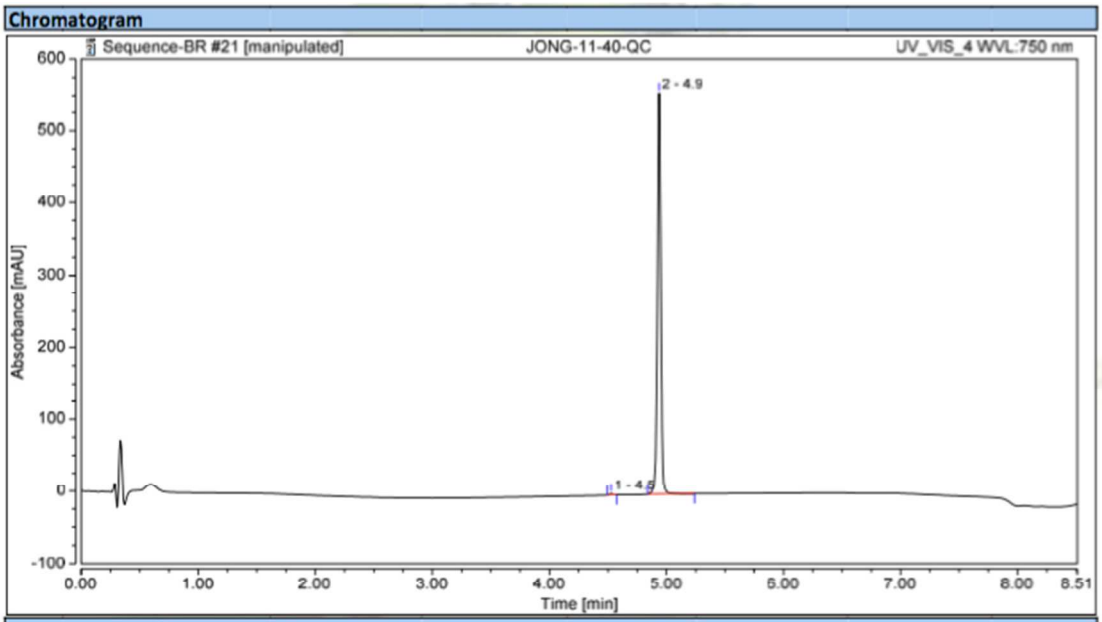
ESI HR mass spectrum (positive mode)



ESI LR mass spectrum (positive mode)



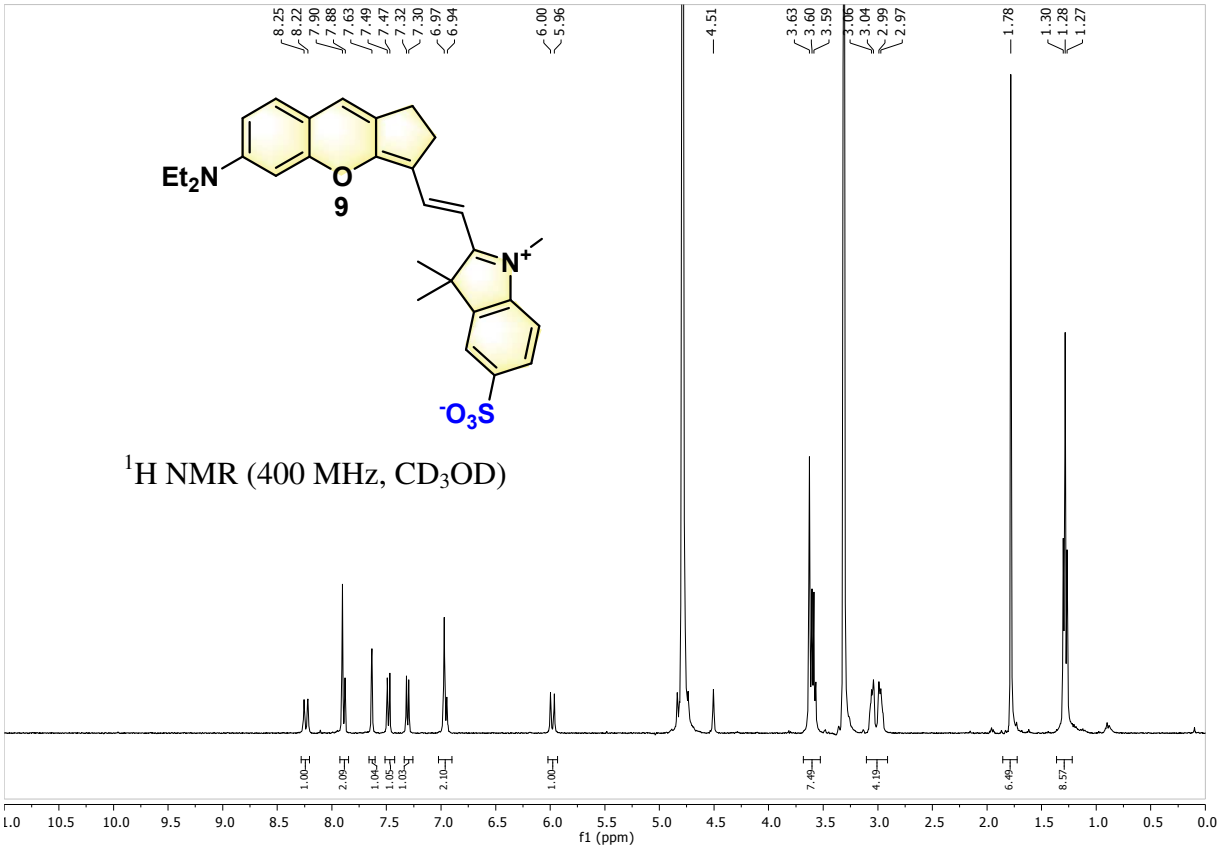
RP-HPLC elution profile (system A, detection at 750 nm)

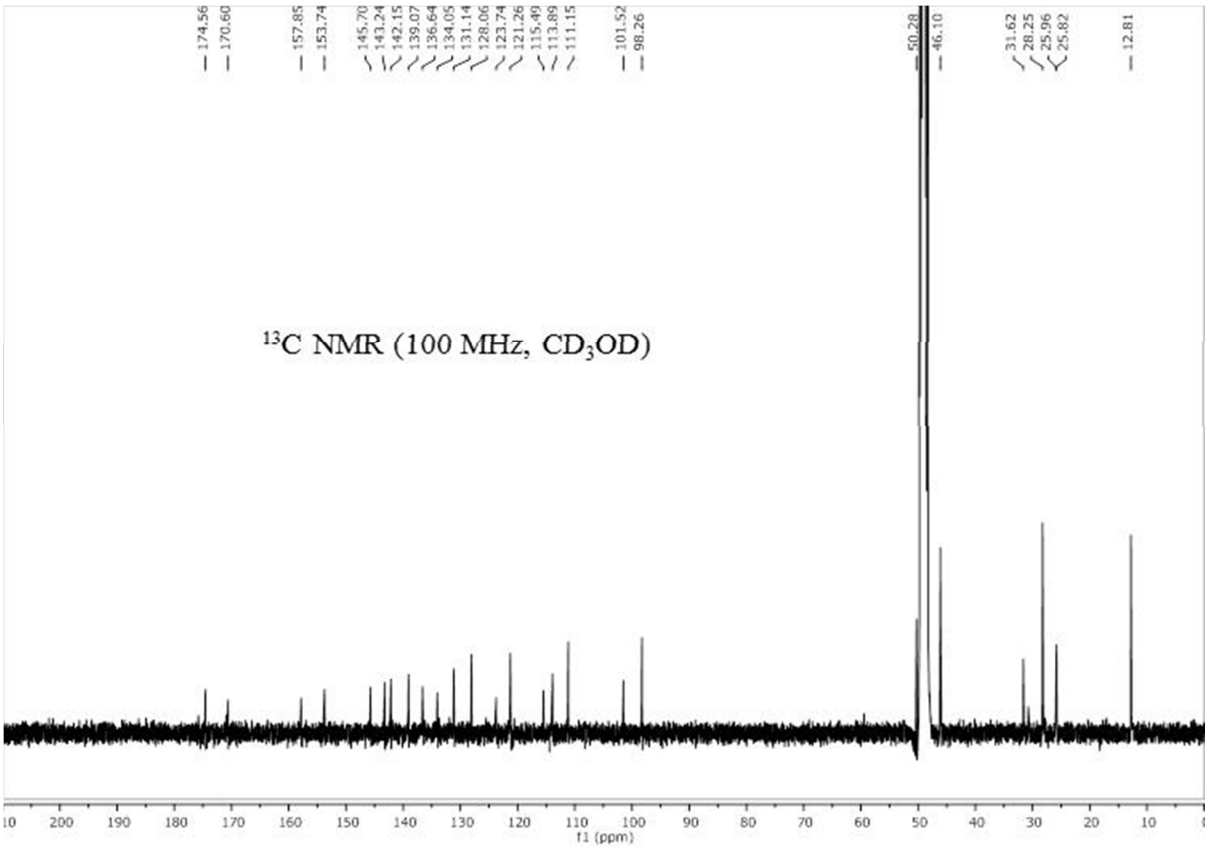


Integration Results

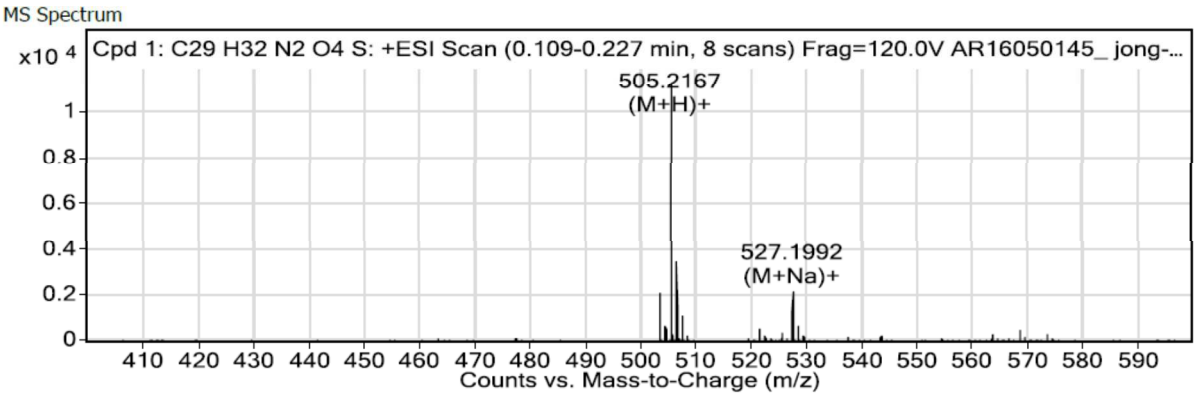
No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		4.527	0.036	1.681	0.19	0.30	n.a.
2		4.937	19.013	558.344	99.81	99.70	n.a.
Total:			**19.048**	**560.025**	**100.00**	**100.00**	

Fluorophore 9

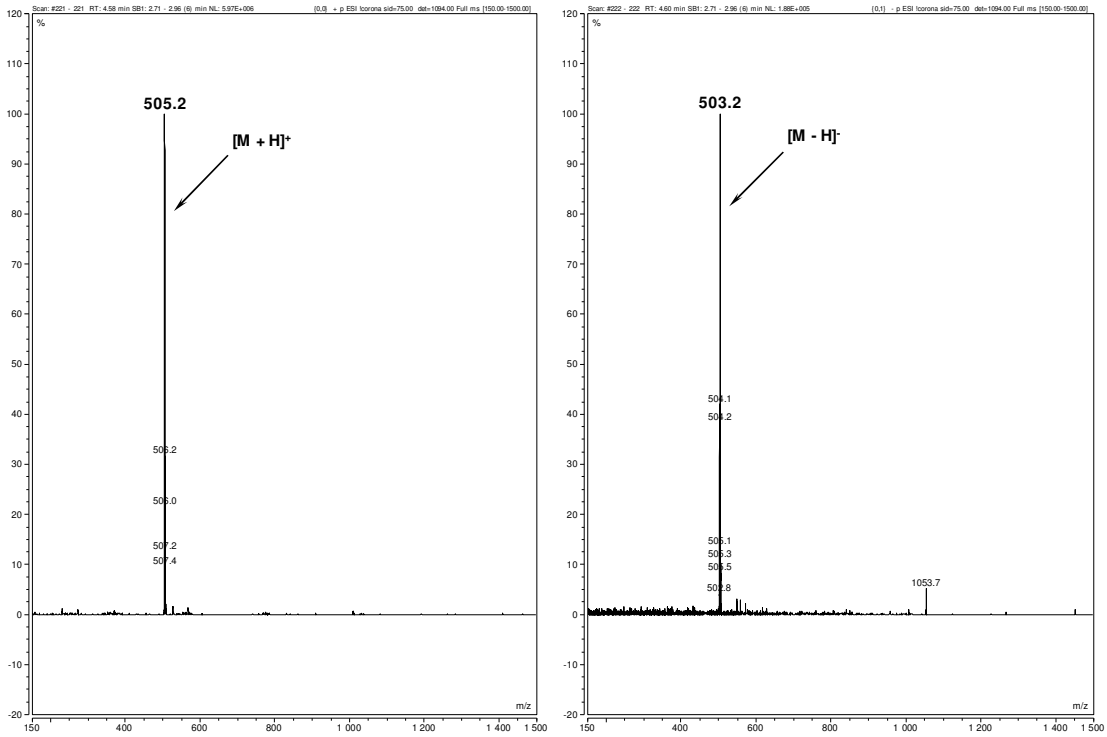




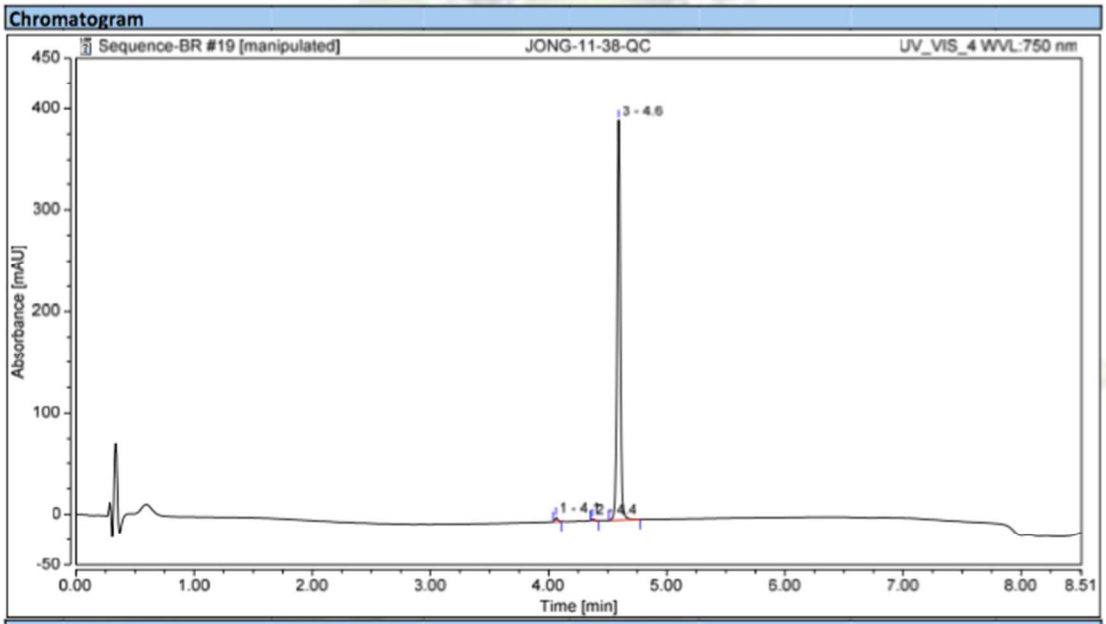
ESI HR mass spectrum (positive mode)



ESI LR mass spectra (positive & negative mode)



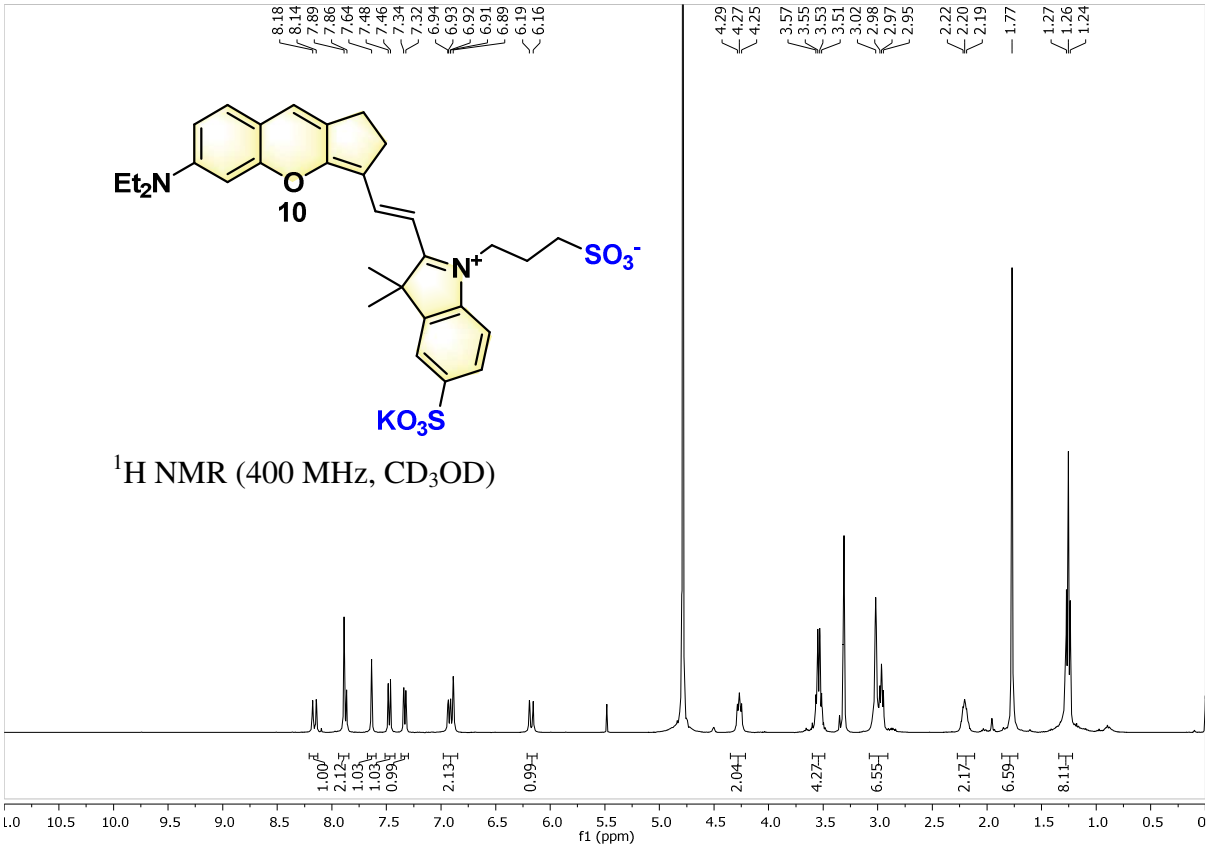
RP-HPLC elution profile (system A, detection at 750 nm)

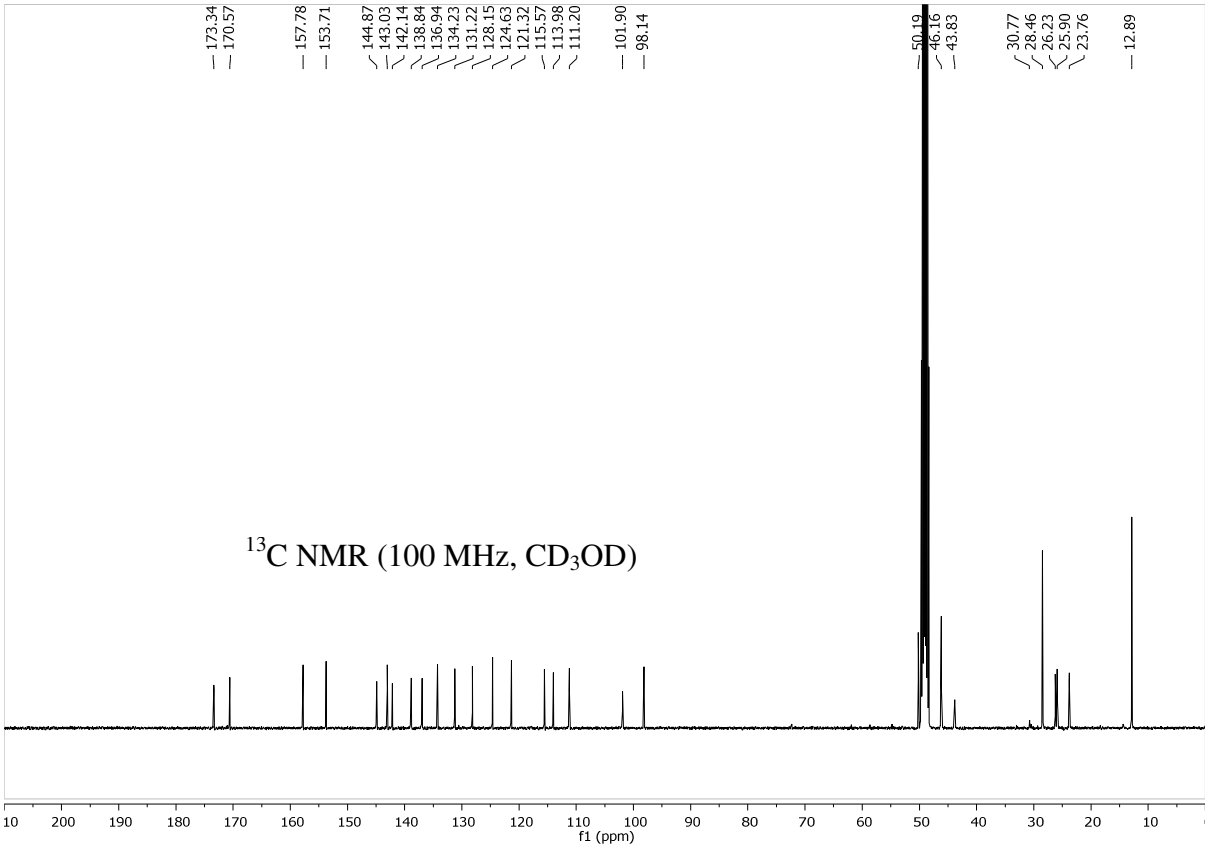


Integration Results

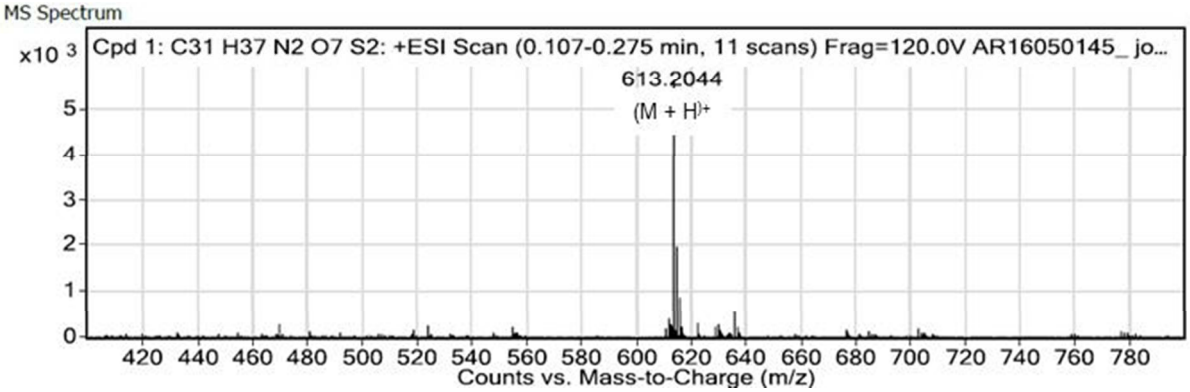
No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		4.063	0.111	4.082	0.89	1.02	n.a.
2		4.373	0.026	1.344	0.21	0.34	n.a.
3		4.593	12.397	394.082	98.91	98.64	n.a.
Total:			**12.534**	**399.508**	**100.00**	**100.00**	

Fluorophore 10

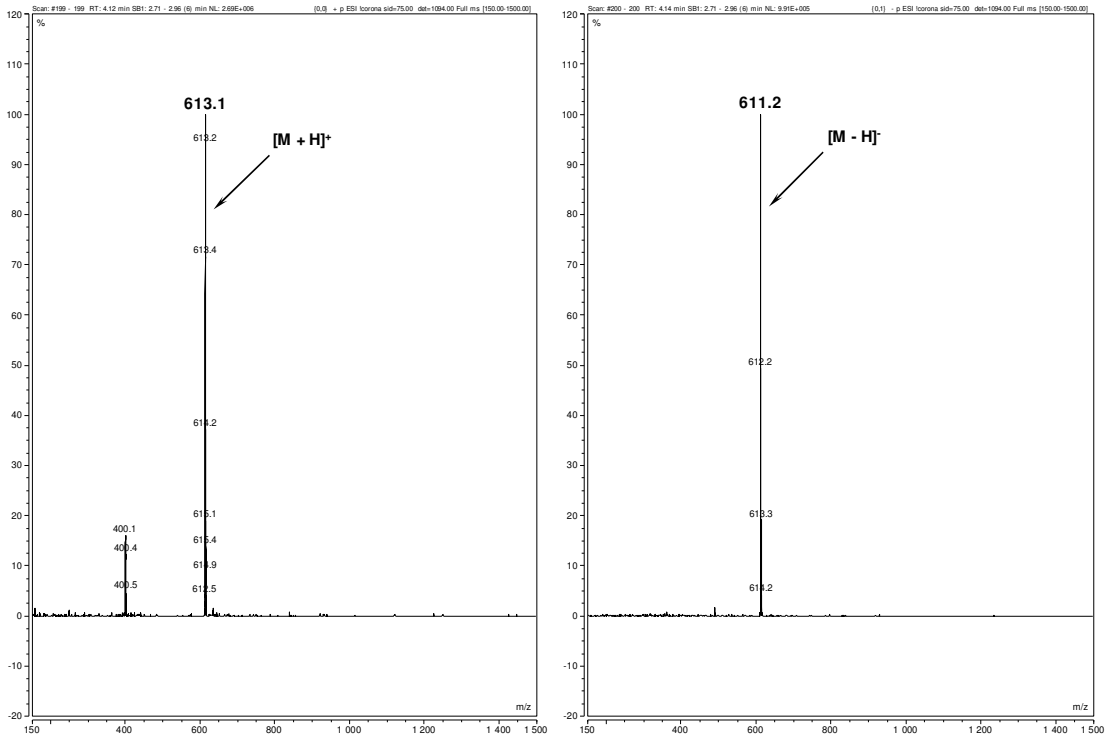




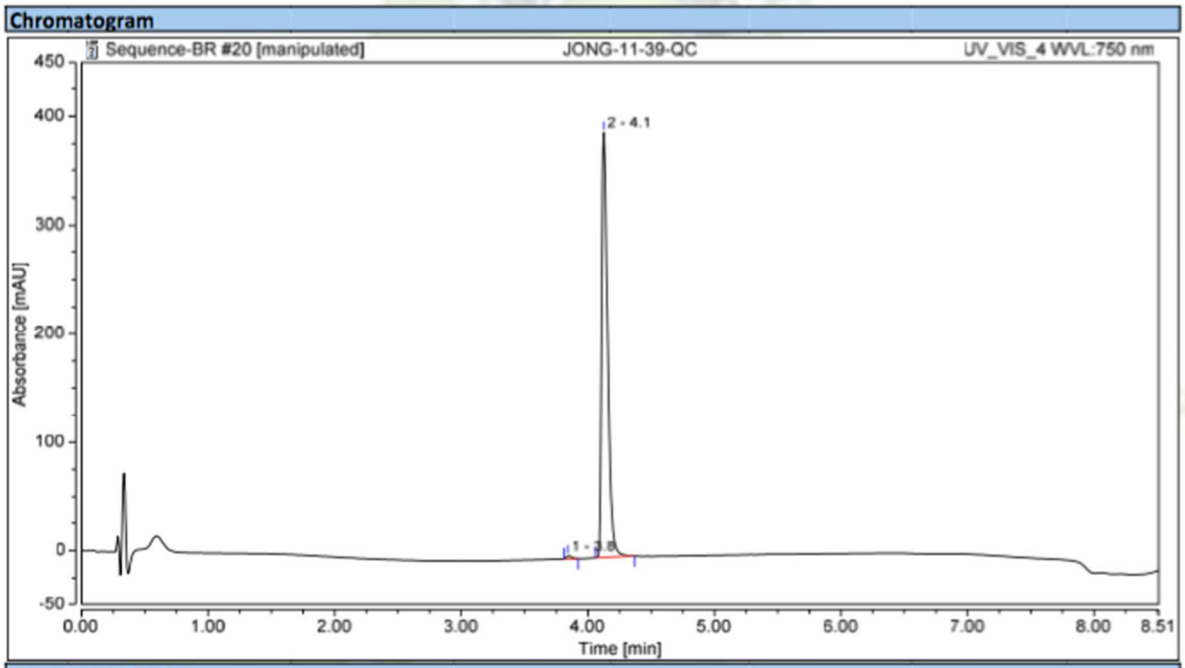
ESI HR mass spectrum (positive mode)



ESI LR mass spectra (positive & negative mode)



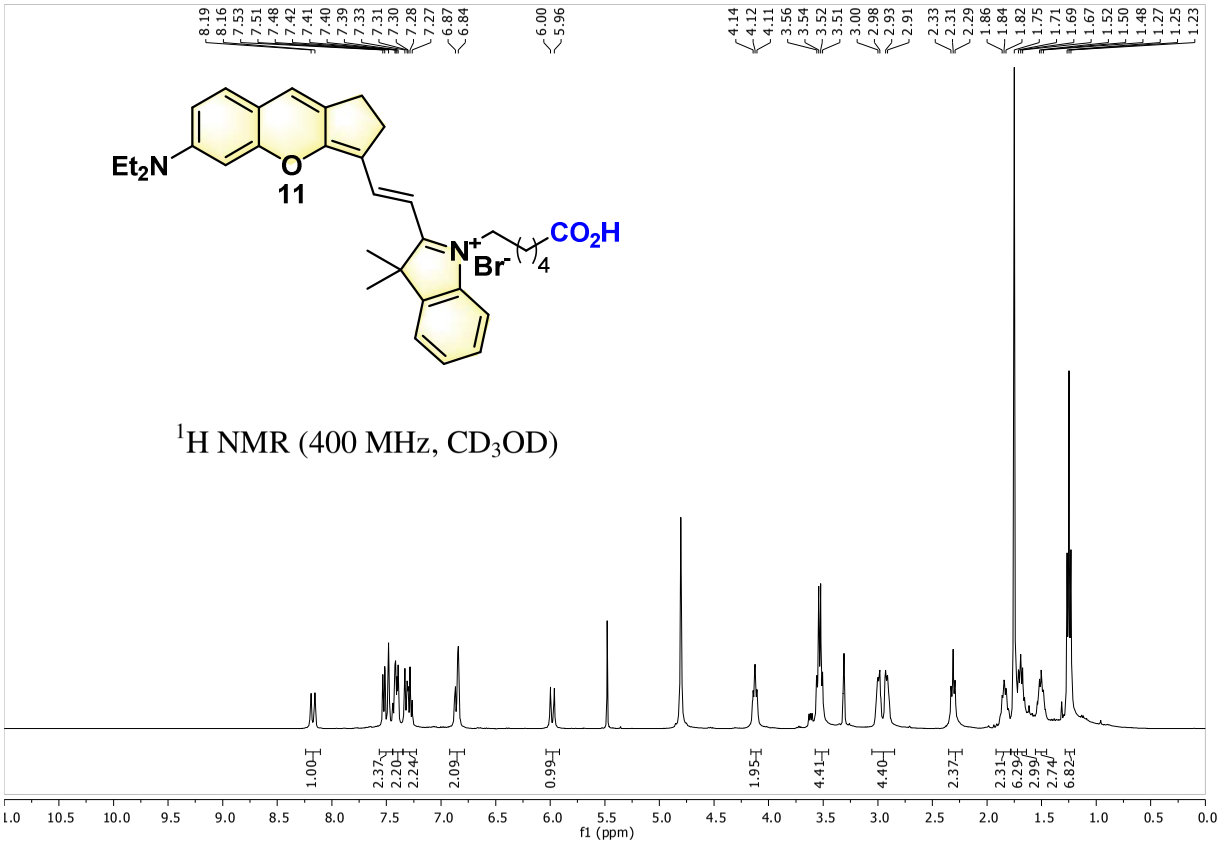
RP-HPLC elution profile (system A, detection at 750 nm)

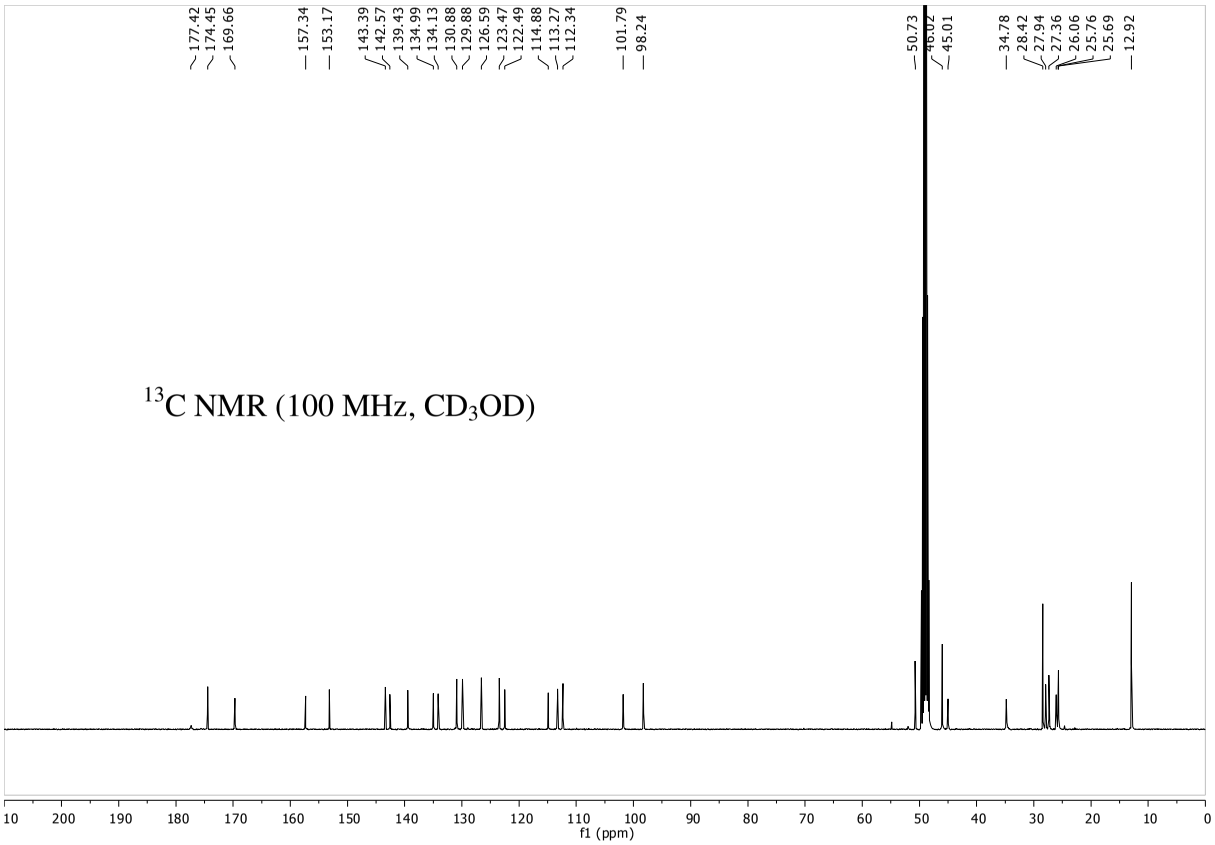


Integration Results

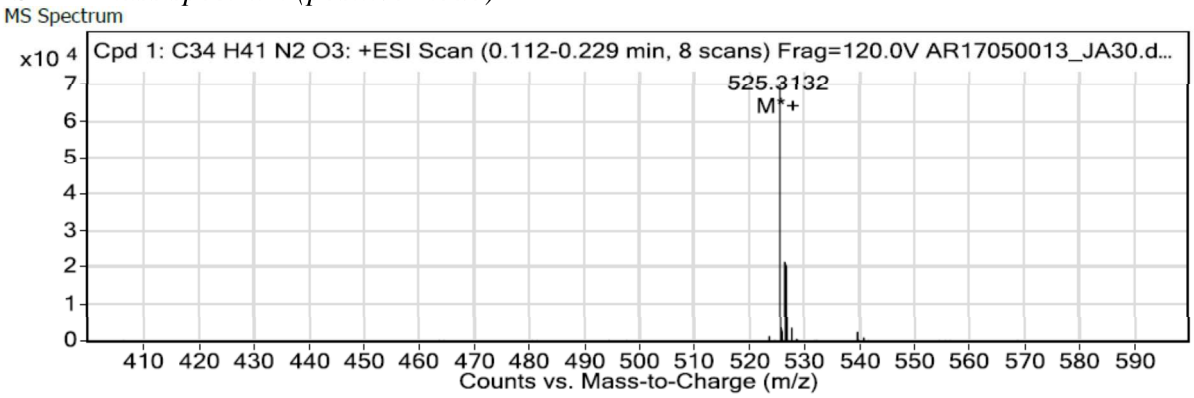
No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		3.843	0.117	2.371	0.54	0.60	n.a.
2		4.127	21.491	391.575	99.46	99.40	n.a.
Total:			**21.608**	**393.946**	**100.00**	**100.00**	

Fluorophore 11

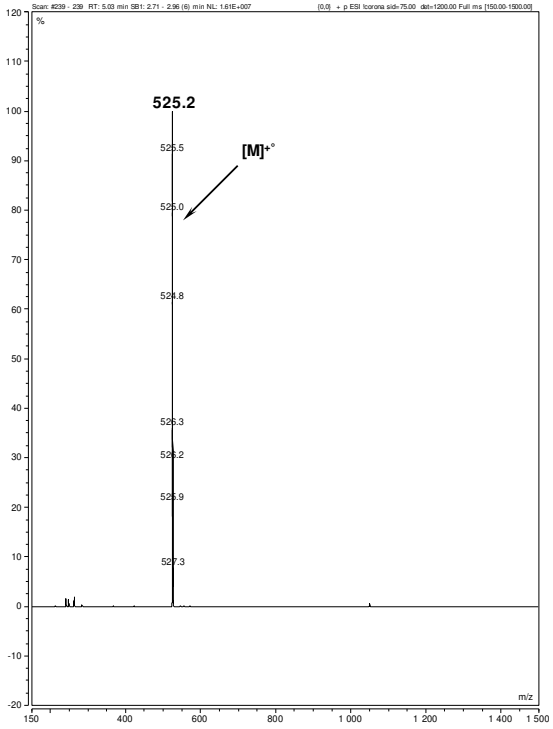




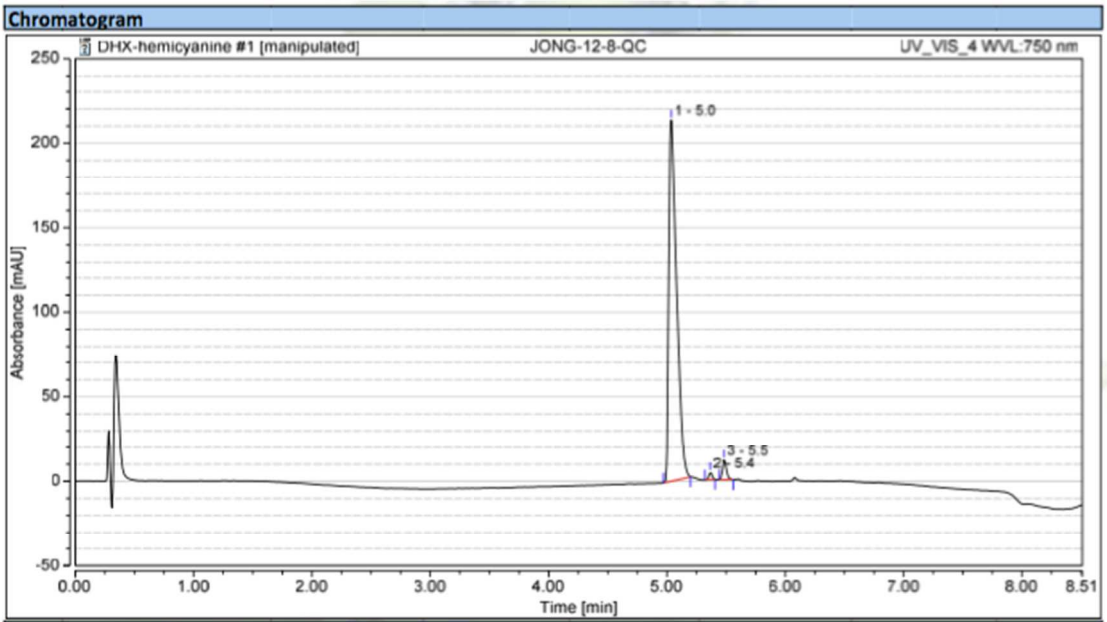
ESI HR mass spectrum (positive mode)



ESI LR mass spectra (positive mode)



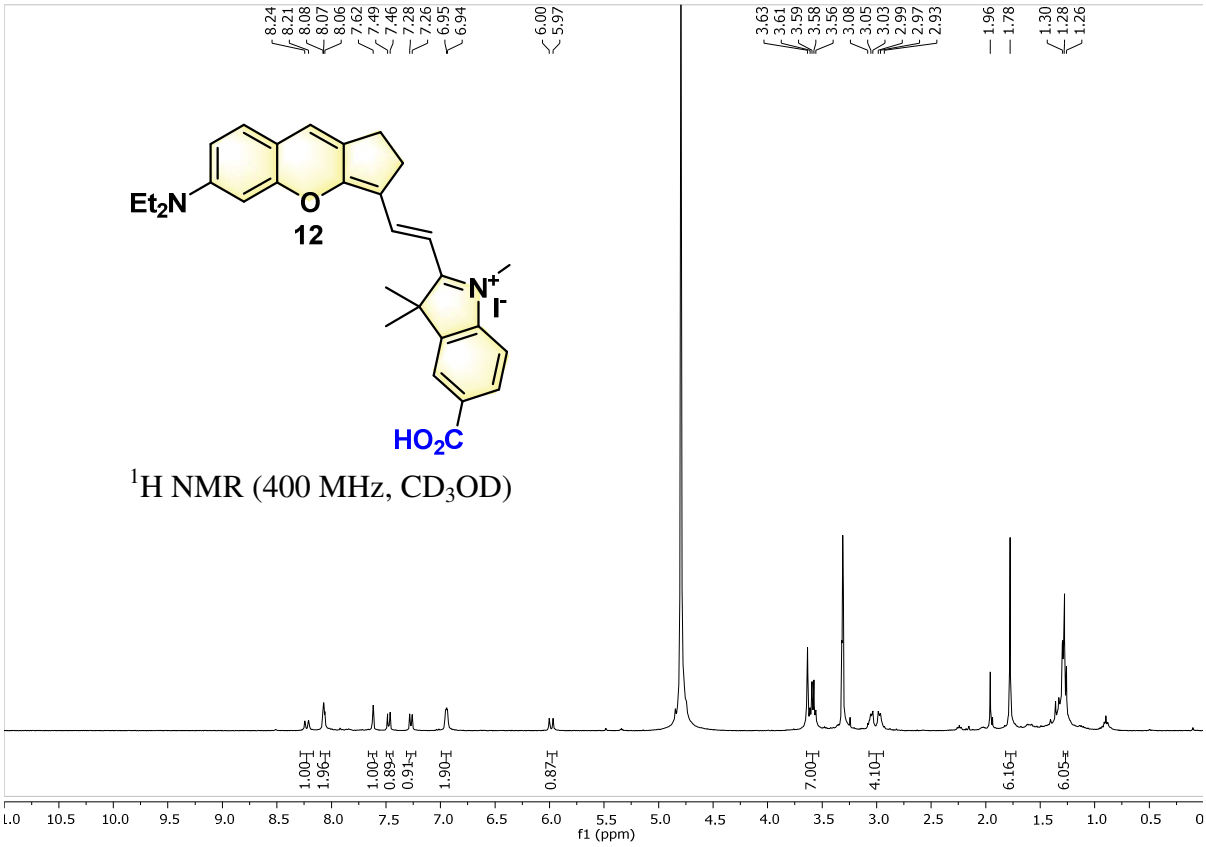
RP-HPLC elution profile (system A, detection at 750 nm)

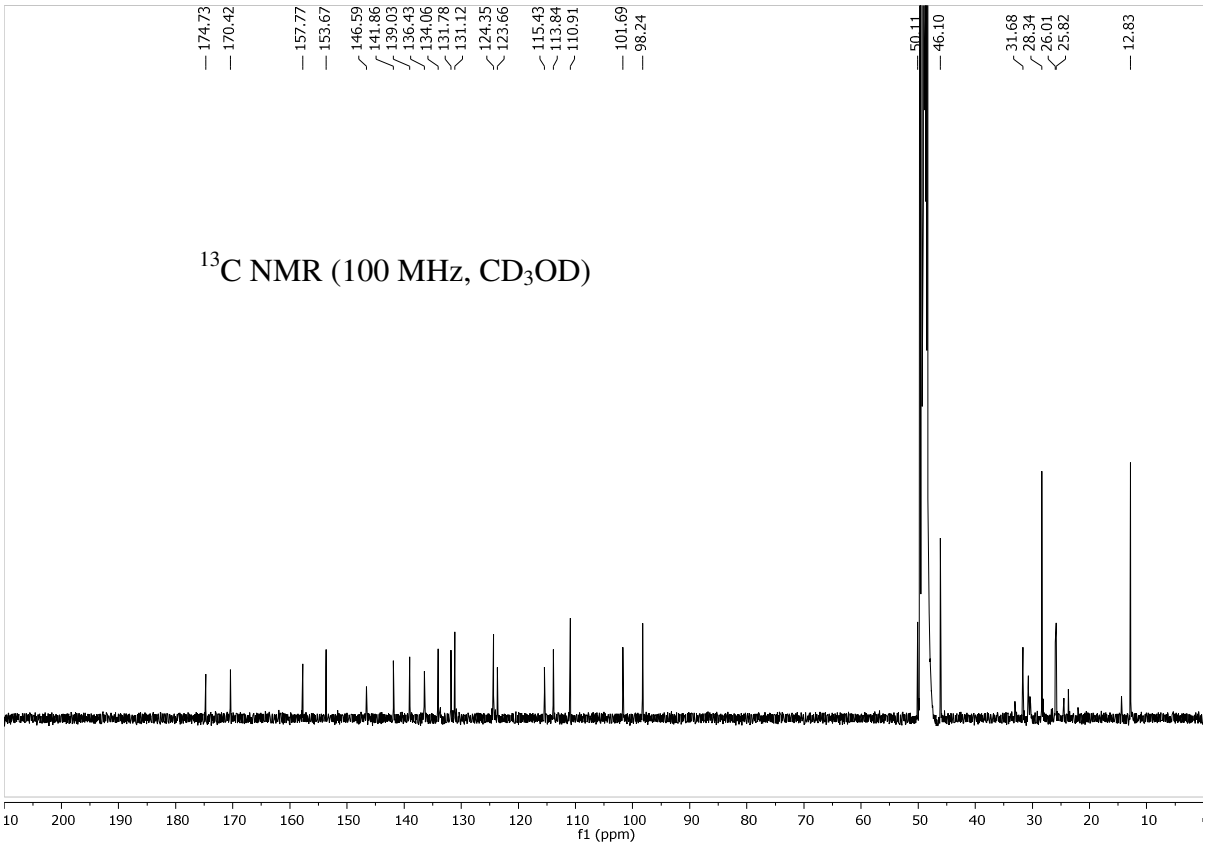


Integration Results

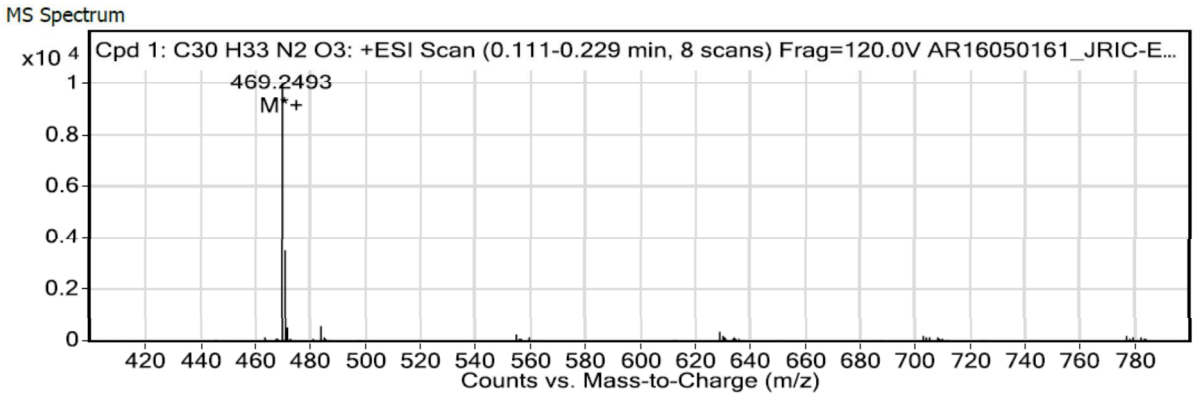
No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		5.037	16.337	213.466	96.74	93.14	n.a.
2		5.367	0.128	4.232	0.76	1.85	n.a.
3		5.483	0.423	11.486	2.50	5.01	n.a.
Total:			**16.888**	**229.184**	**100.00**	**100.00**	

Fluorophore 12

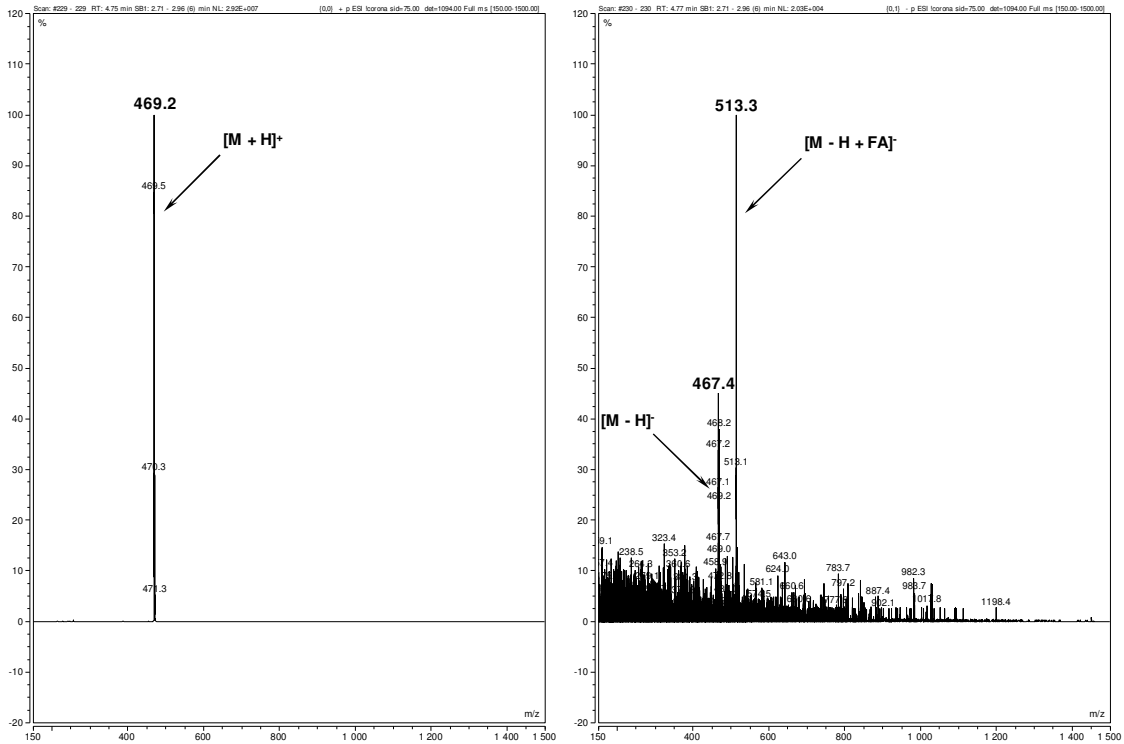




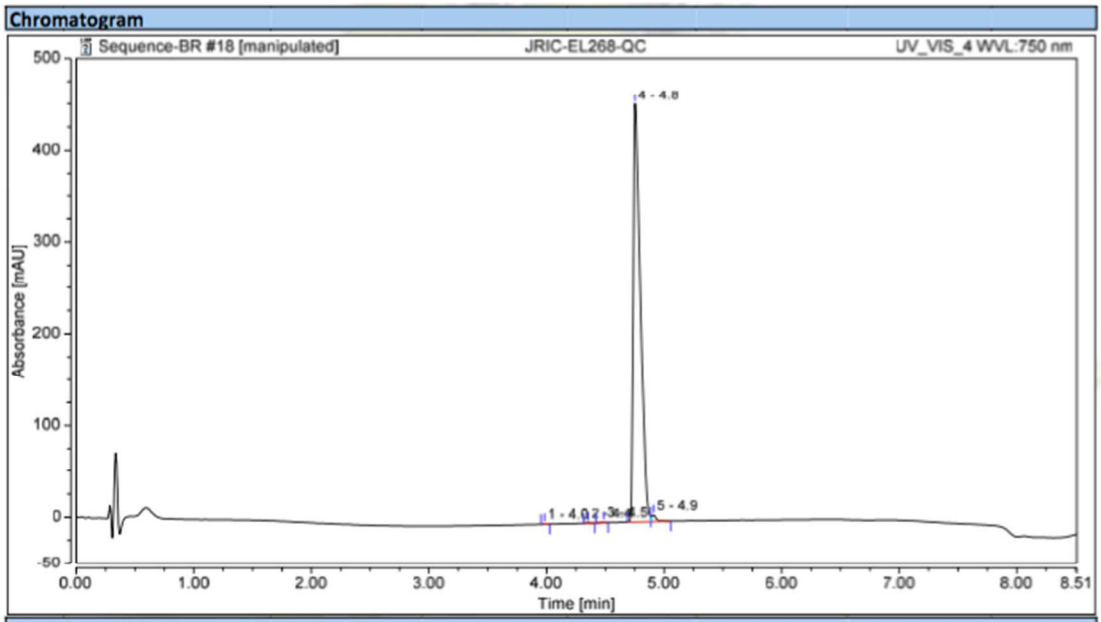
ESI HR mass spectrum (positive mode)



ESI LR mass spectra (positive & negative mode)



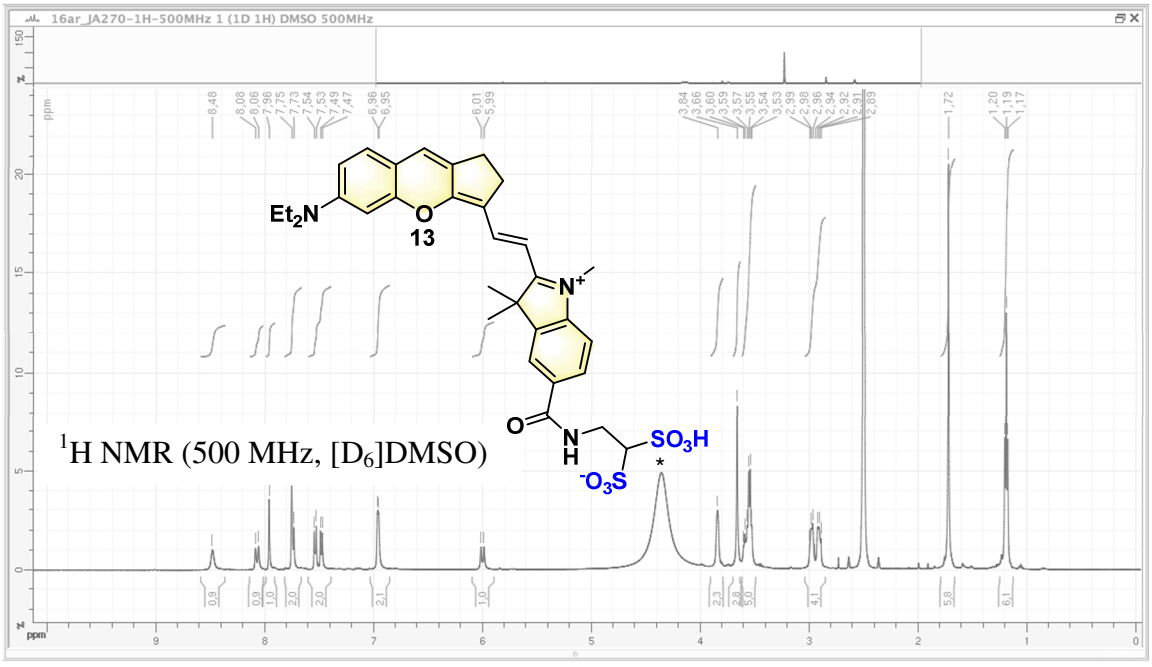
RP-HPLC elution profile (system A, detection at 750 nm)



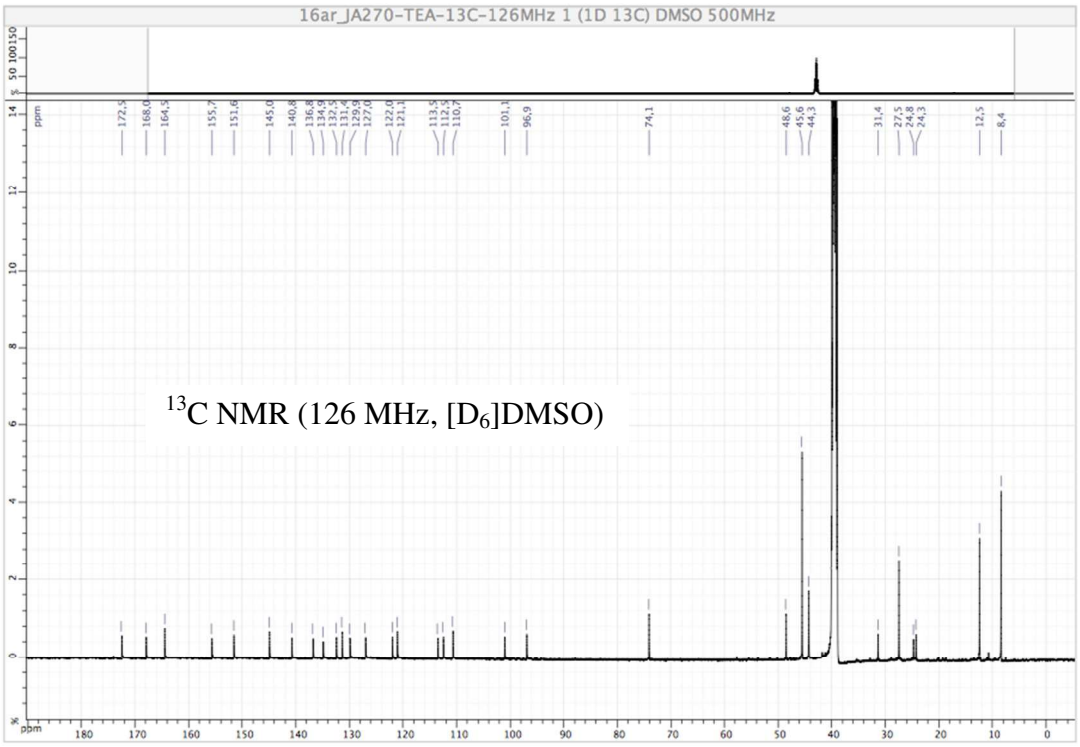
Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		3.983	0.014	0.535	0.04	0.12	n.a.
2		4.353	0.021	0.744	0.07	0.16	n.a.
3		4.487	0.039	0.871	0.12	0.19	n.a.
4		4.753	31.826	455.236	98.76	98.01	n.a.
5		4.913	0.325	7.114	1.01	1.53	n.a.
Total:			**32.225**	**464.500**	**100.00**	**100.00**	

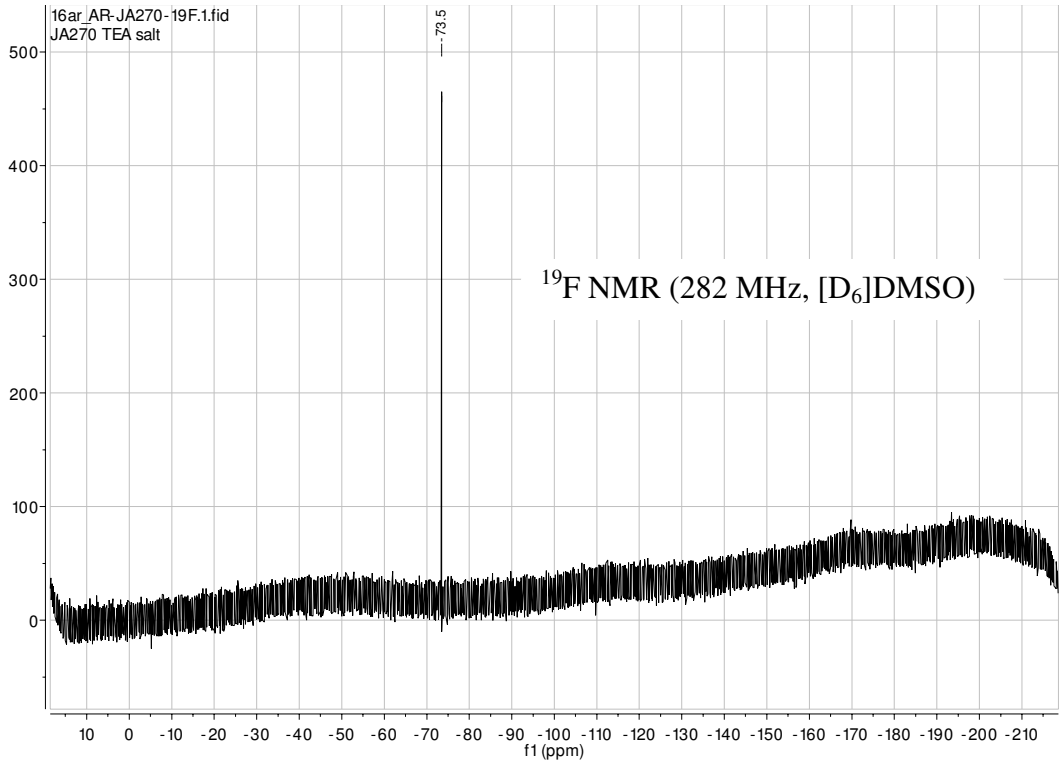
Fluorophore 13



**broad peak assigned to water (found in [D_6]DMSO)*

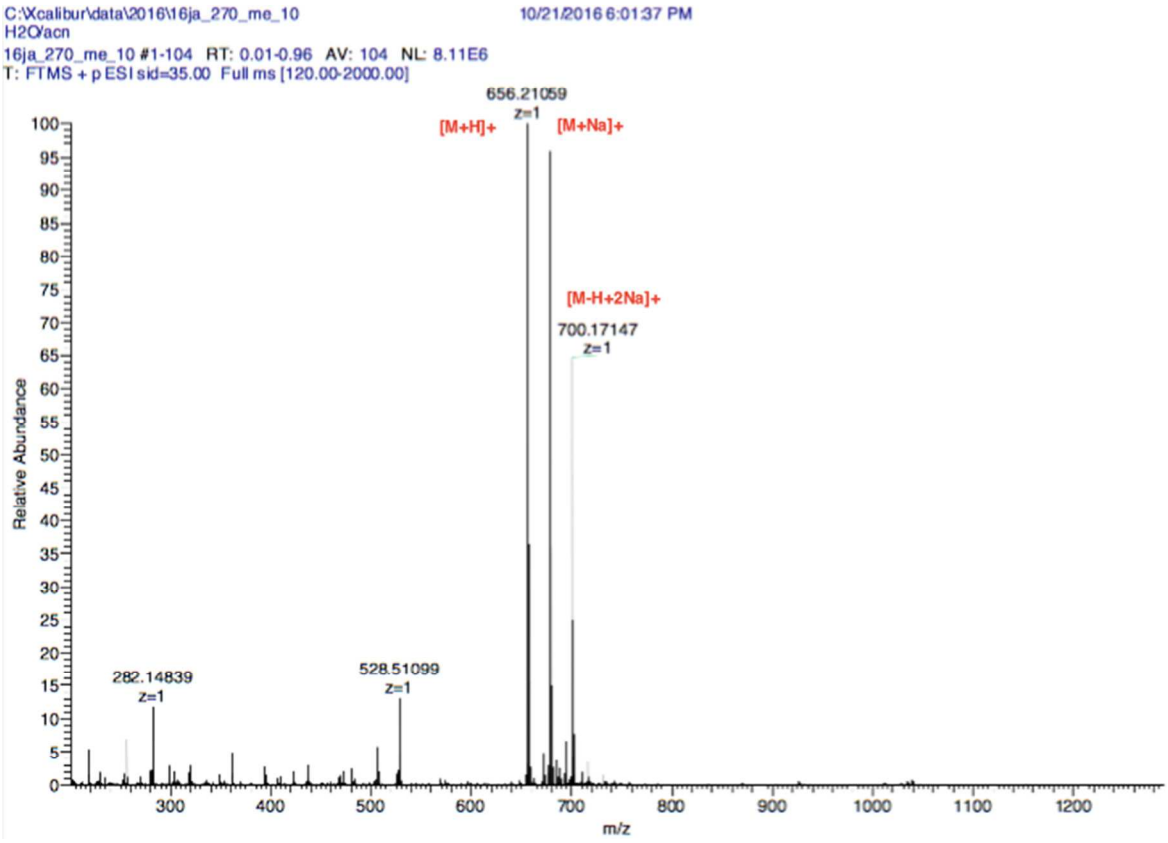


^{13}C NMR (126 MHz, [D$_6$]DMSO)

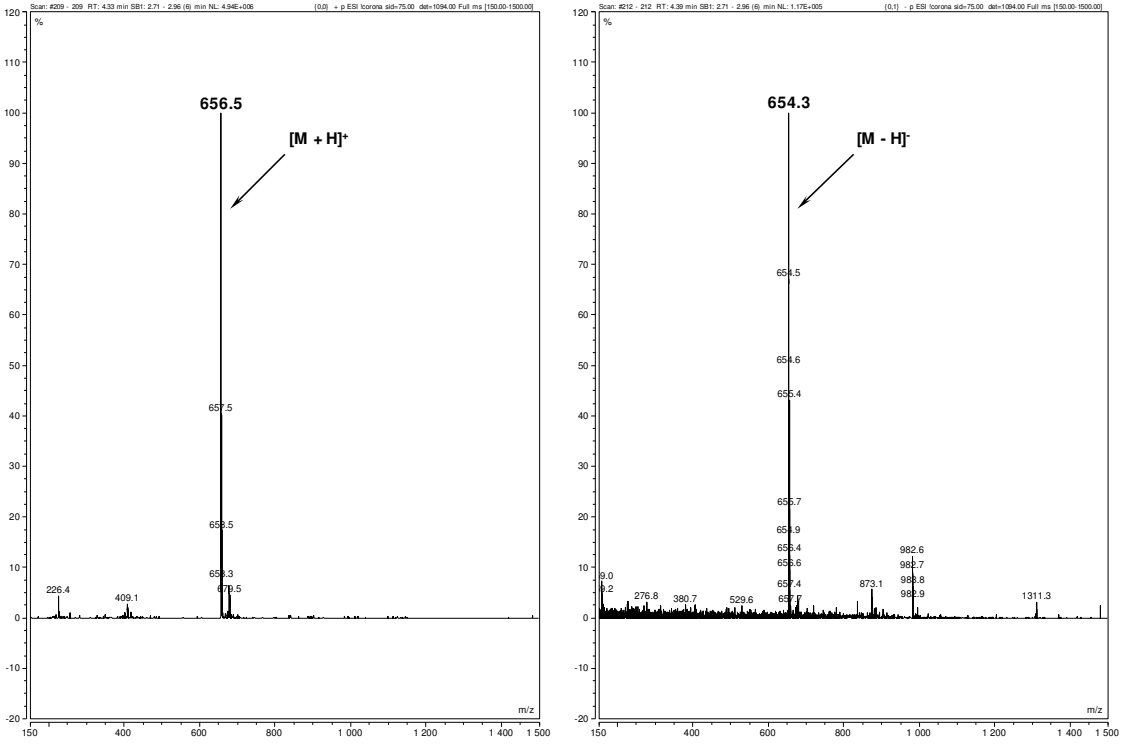


^{19}F NMR (282 MHz, [D$_6$]DMSO)

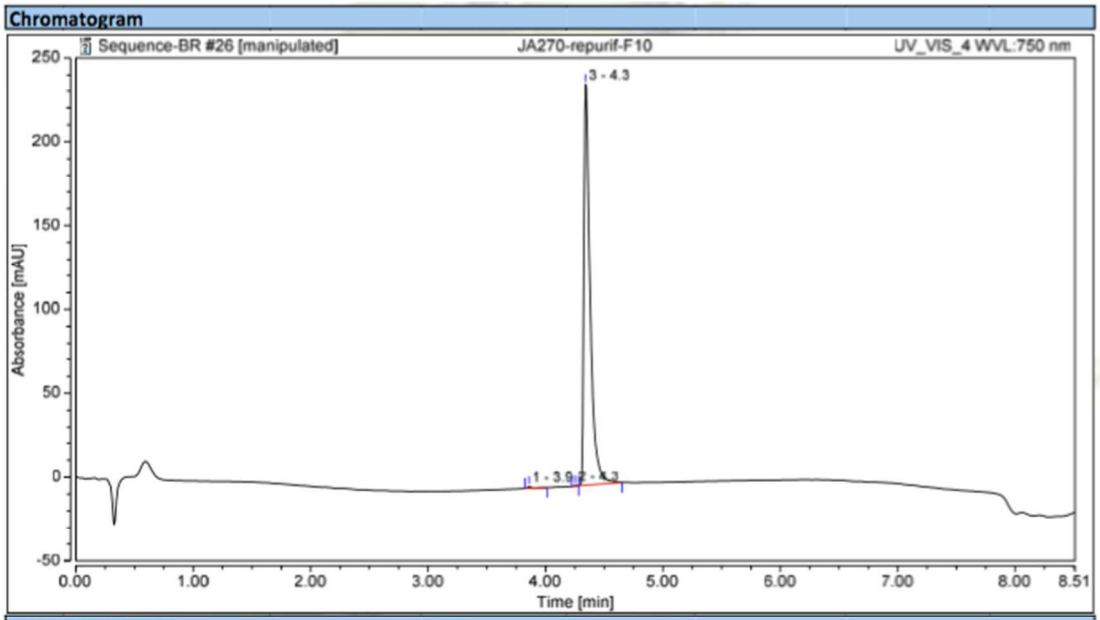
ESI HR mass spectrum (positive mode)



ESI LR mass spectra (positive & negative mode)



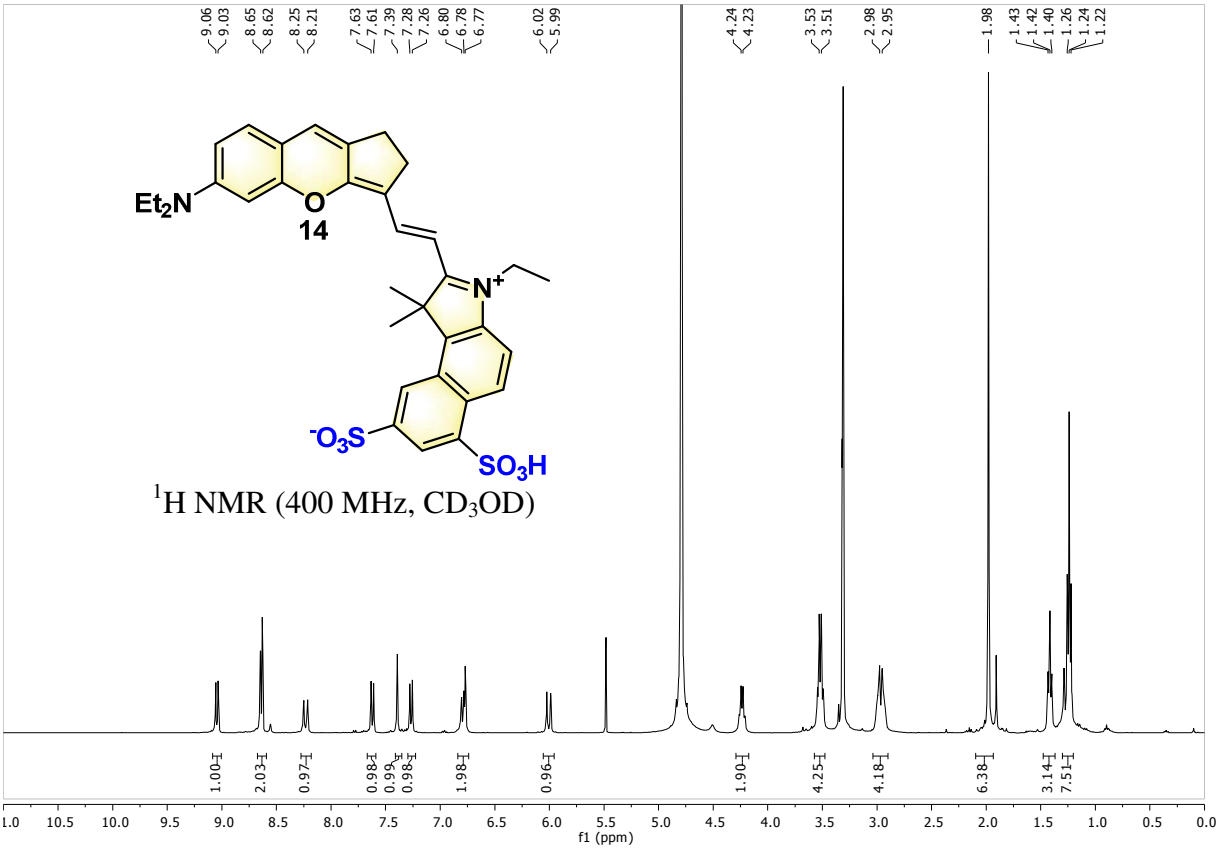
RP-HPLC elution profile (system A, detection at 750 nm)

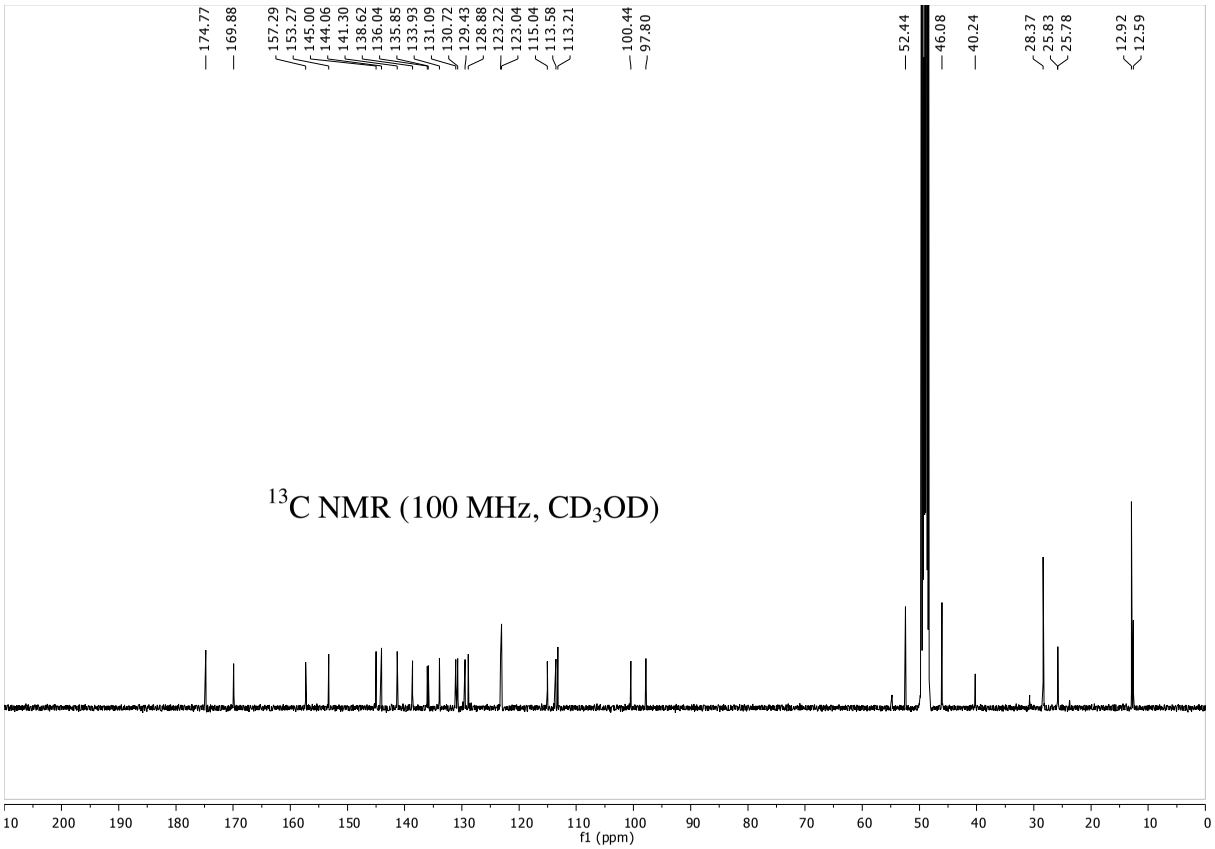


Integration Results

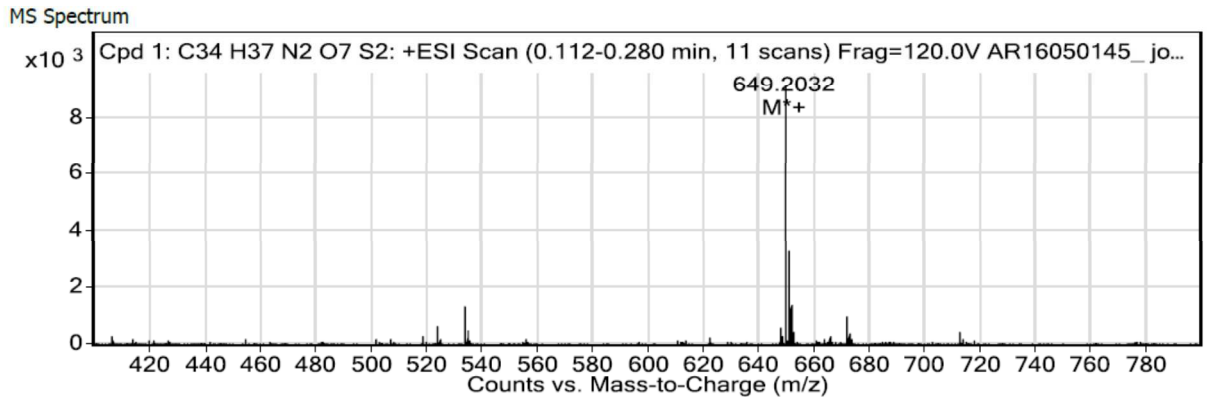
No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		3.860	0.036	1.041	0.25	0.43	n.a.
2		4.253	0.012	0.392	0.08	0.16	n.a.
3		4.343	14.680	239.247	99.67	99.40	n.a.
Total:			**14.728**	**240.679**	**100.00**	**100.00**	

Fluorophore 14

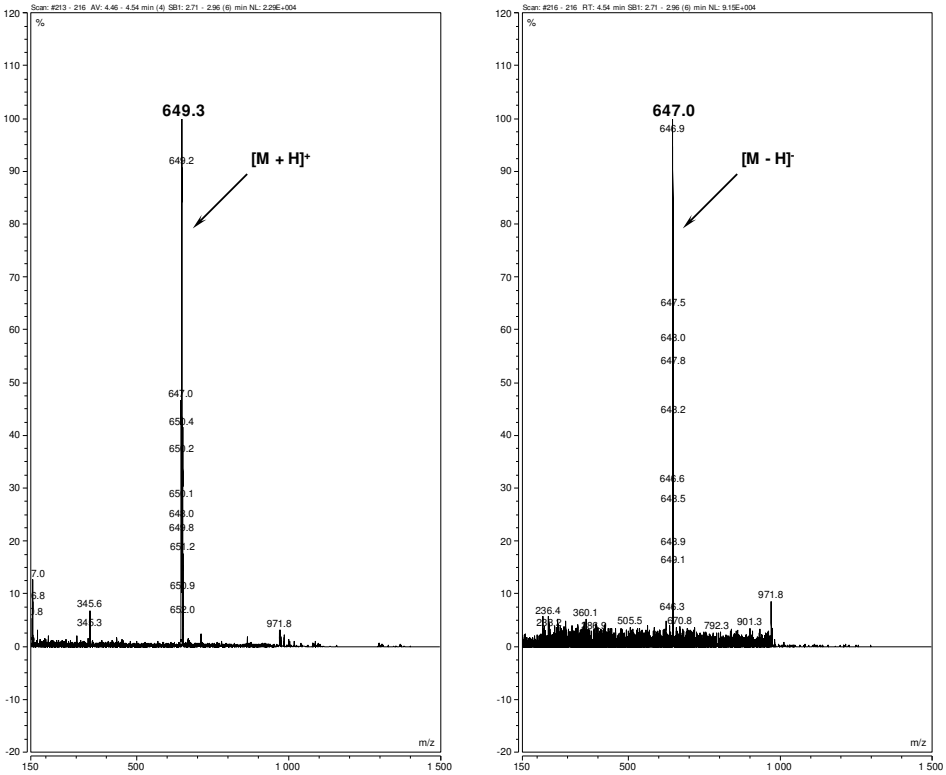




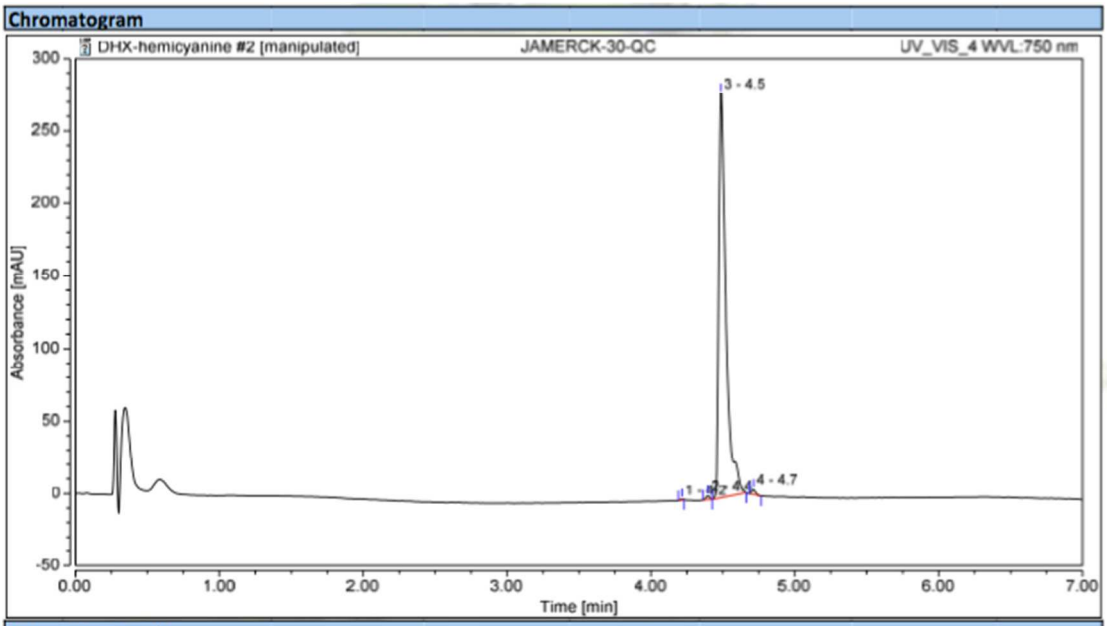
ESI HR mass spectrum (positive mode)



ESI LR mass spectra (positive & negative mode)



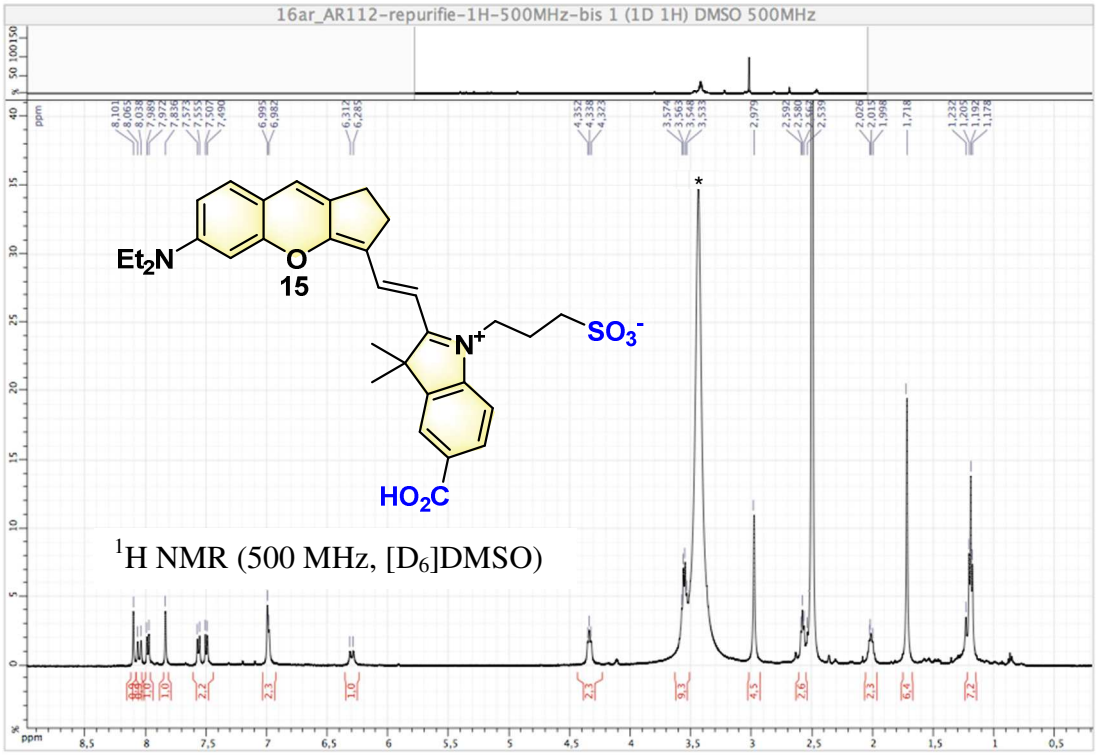
RP-HPLC elution profile (system A, detection at 750 nm)



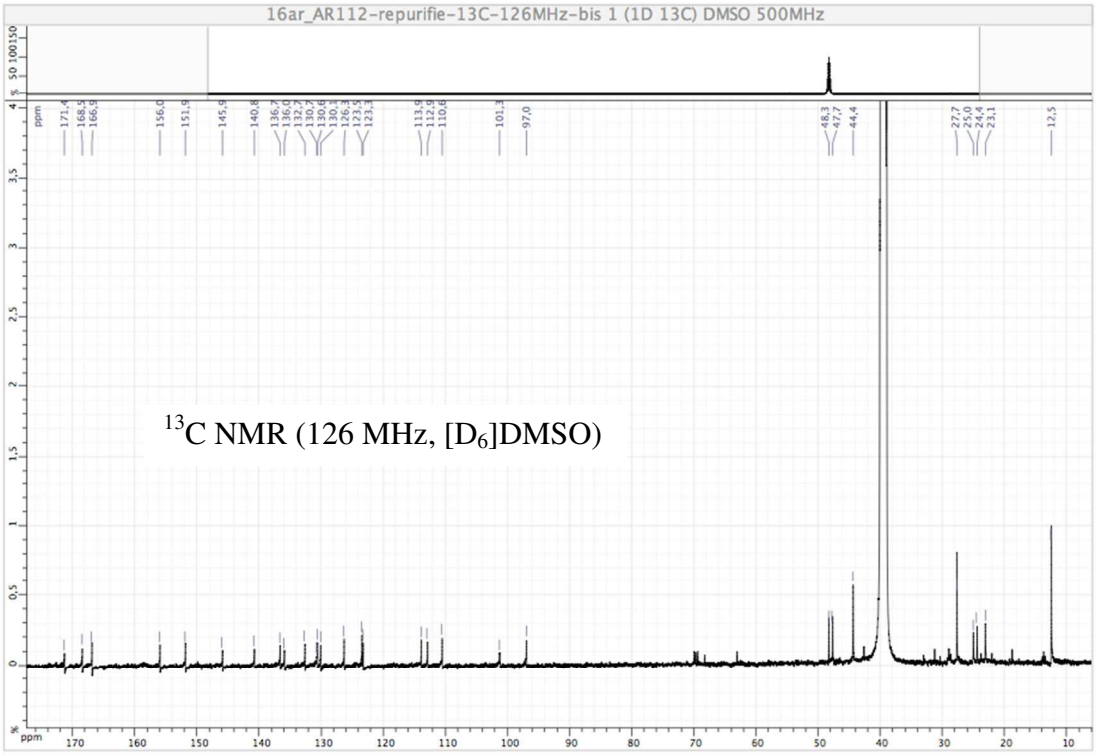
Integration Results

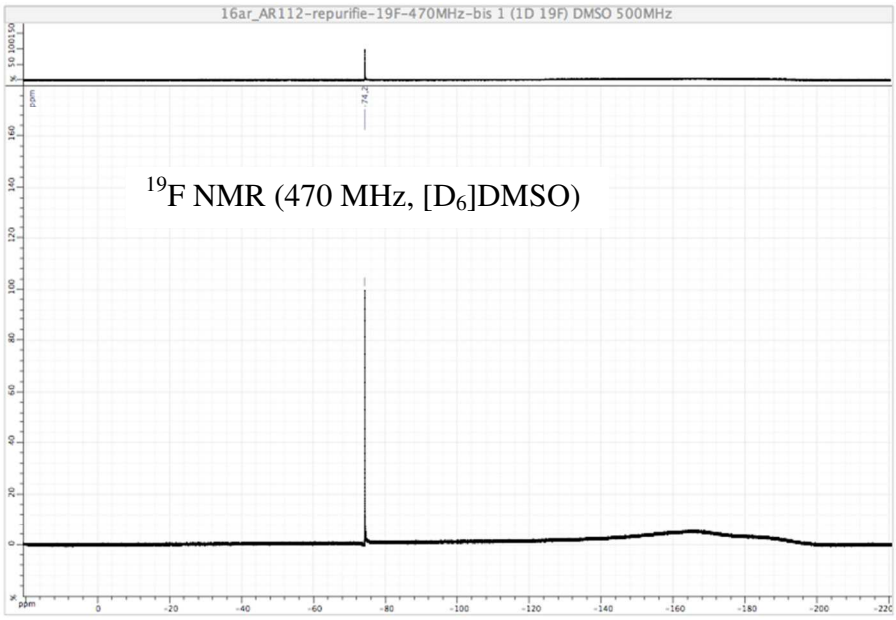
No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		4.217	0.011	0.521	0.07	0.18	n.a.
2		4.397	0.081	2.735	0.50	0.96	n.a.
3		4.487	16.146	279.008	98.83	97.70	n.a.
4		4.713	0.099	3.315	0.61	1.16	n.a.
Total:			**16.337**	**285.579**	**100.00**	**100.00**	

Fluorophore 15



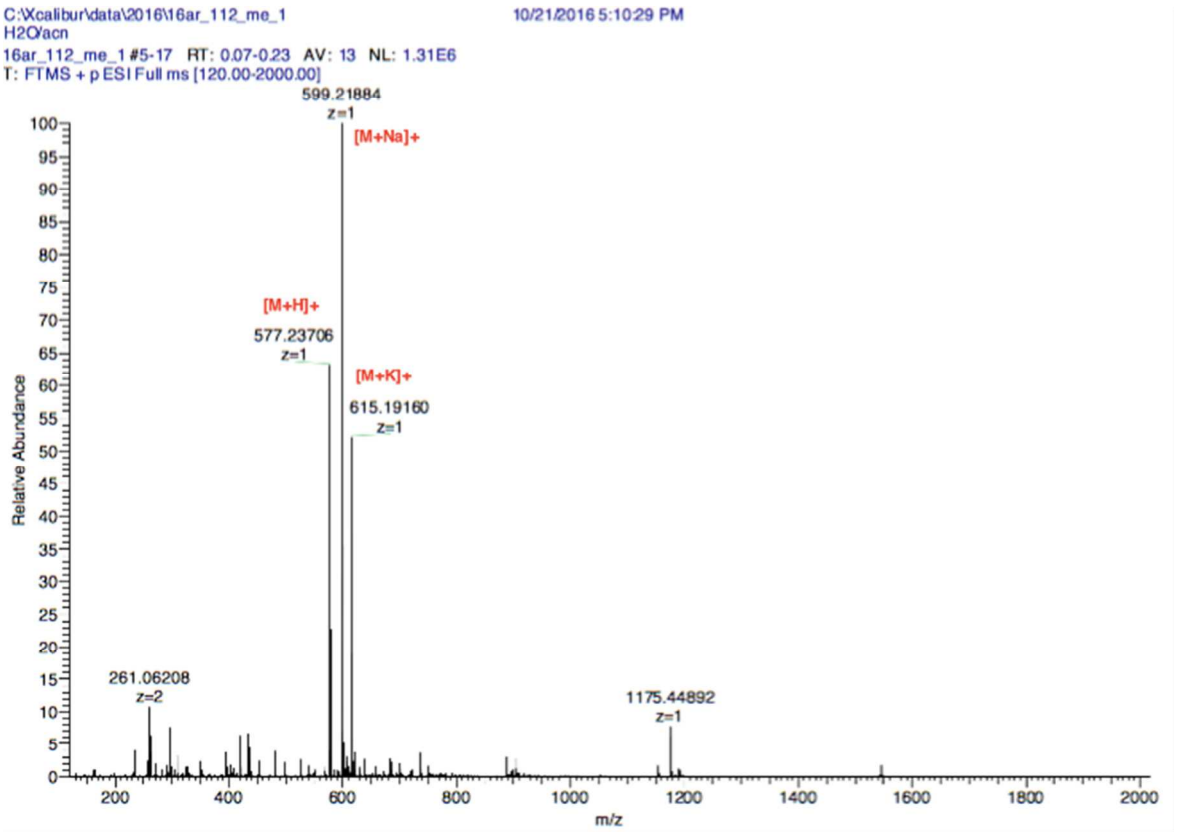
broad peak assigned to water (found in [D_6]DMSO)



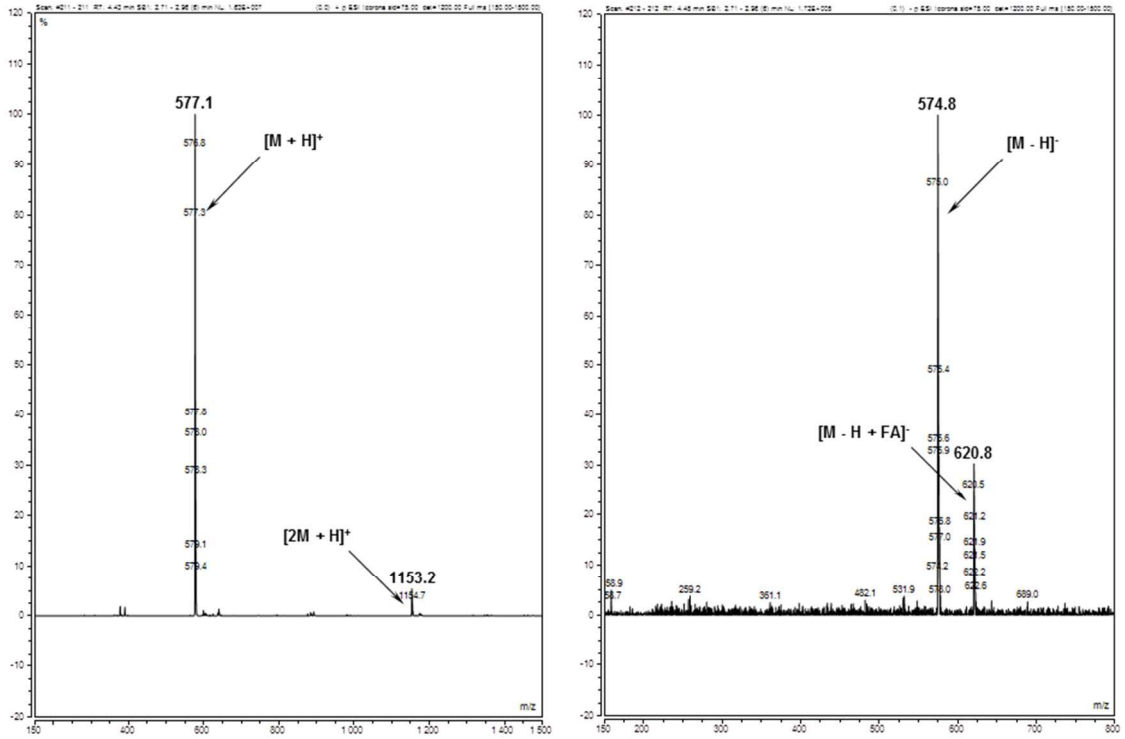


^{19}F NMR (470 MHz, [D_6]DMSO)

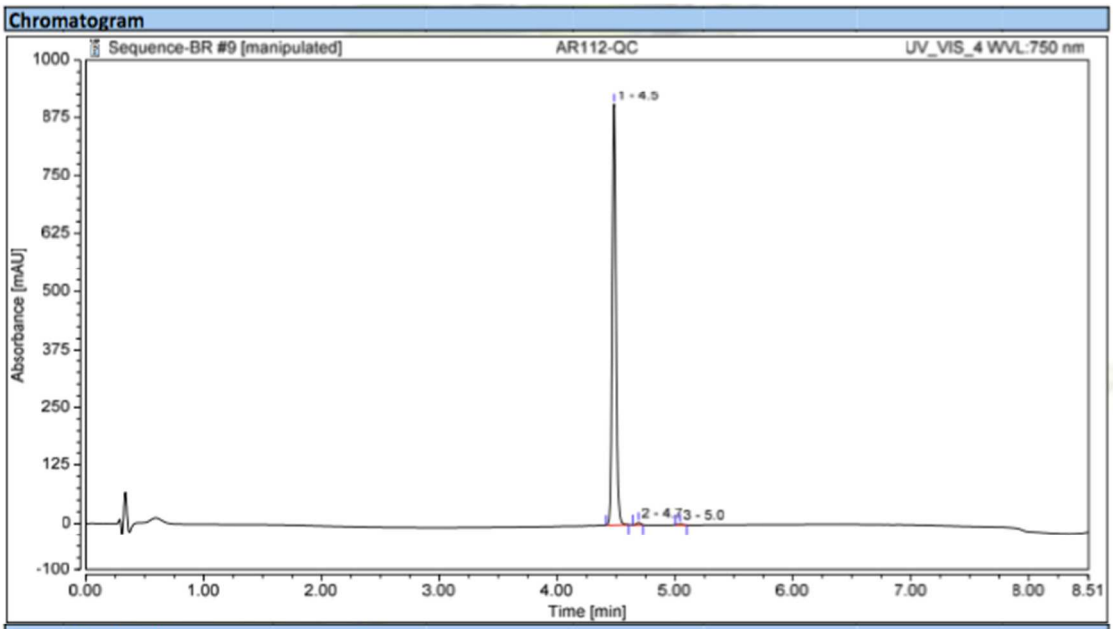
ESI HR mass spectrum (positive mode)



ESI LR mass spectra (positive & negative mode)



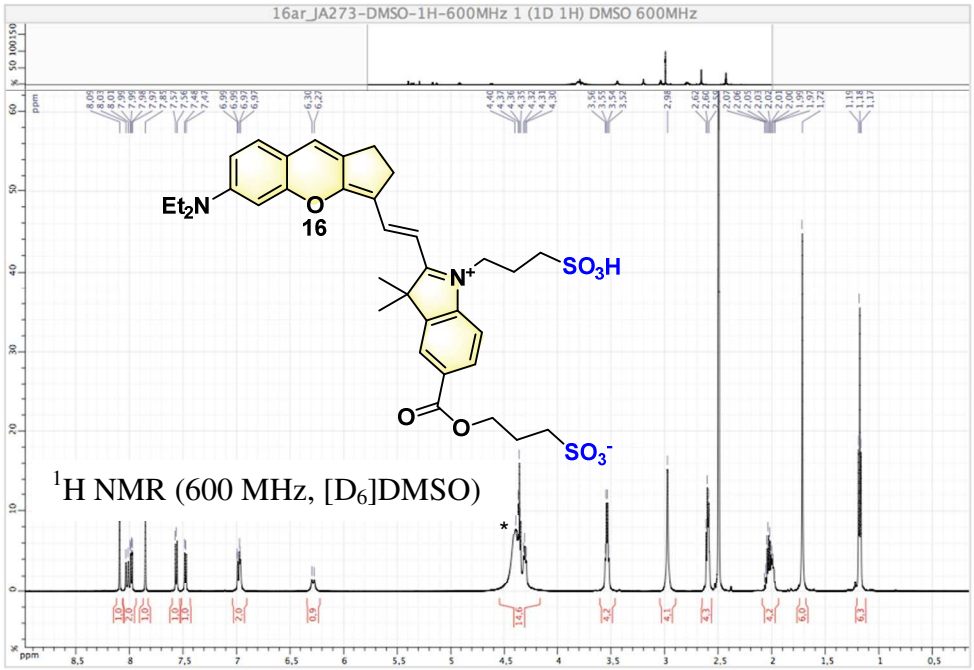
RP-HPLC elution profile (system A, detection at 750 nm)



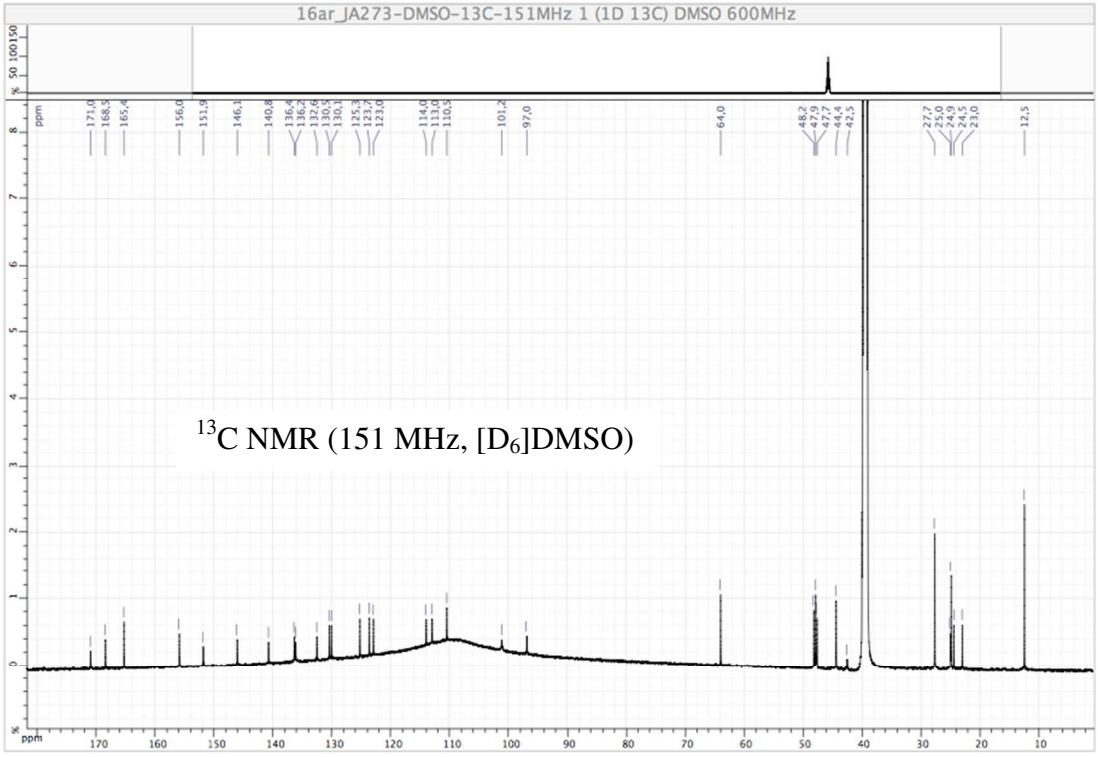
Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		4.483	33.943	906.806	99.35	99.35	n.a.
2		4.690	0.176	4.993	0.52	0.55	n.a.
3		5.043	0.047	0.969	0.14	0.11	n.a.
Total:			**34.167**	**912.767**	**100.00**	**100.00**	

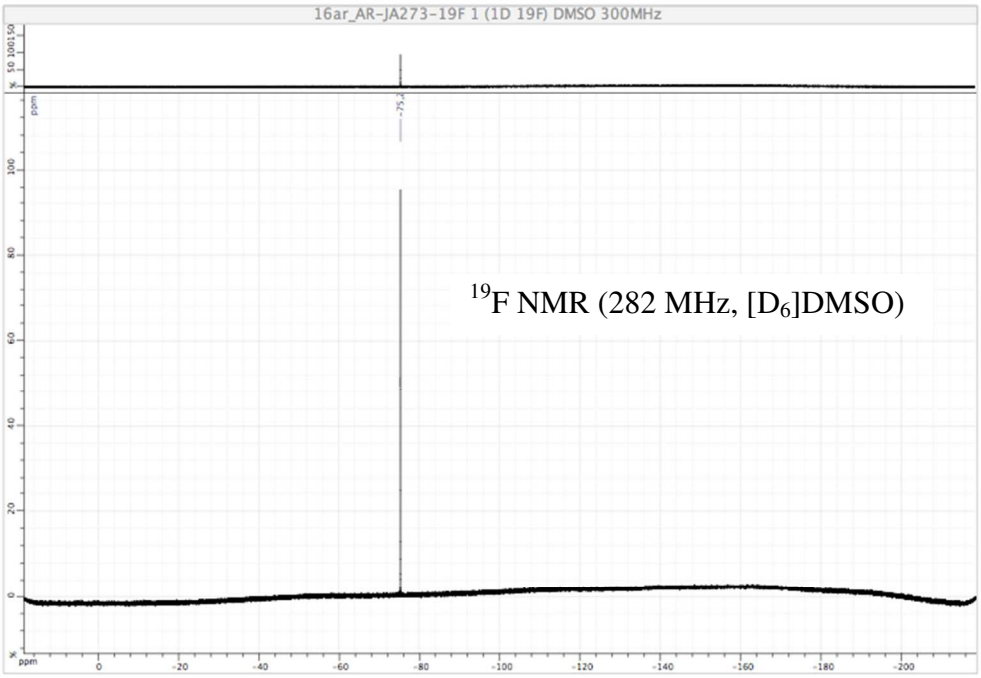
Fluorophore 16



**broad peak assigned to water (found in [D_6]DMSO)*

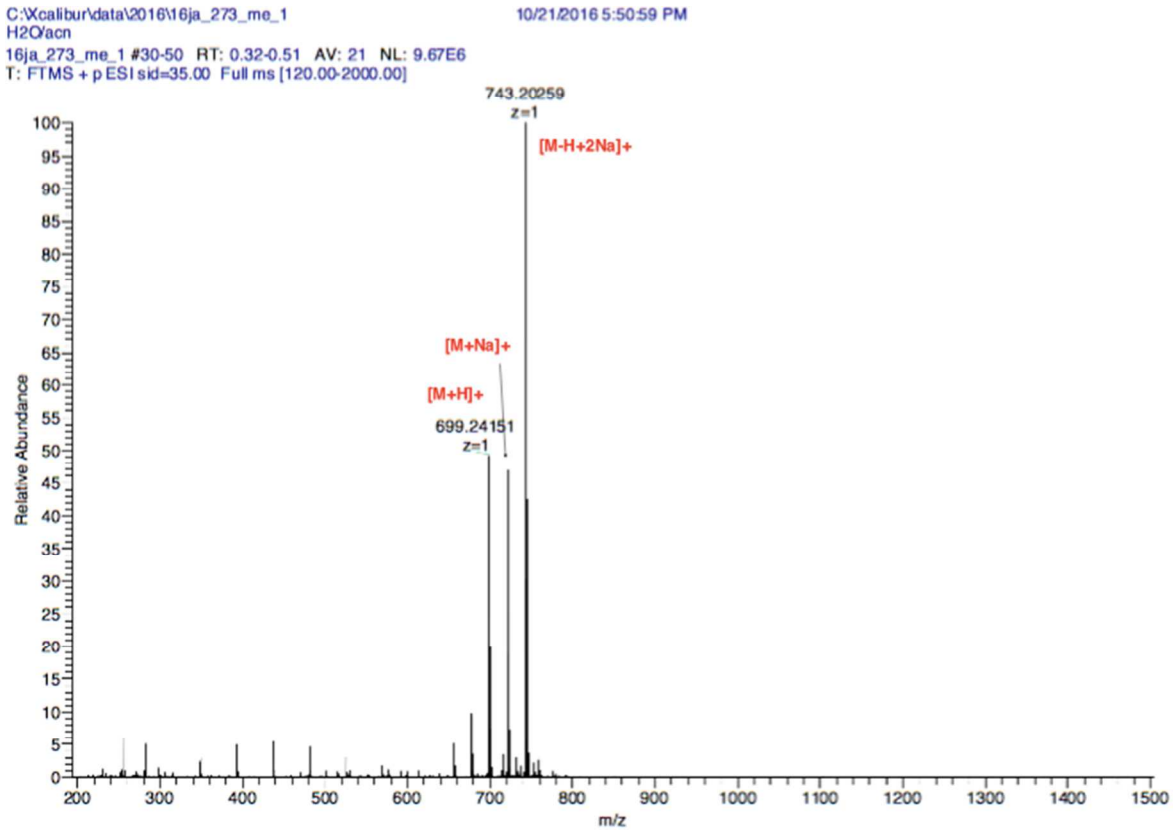


**bump assigned to glue used for assembly of inner ^{1}H coil (BBI probe)*

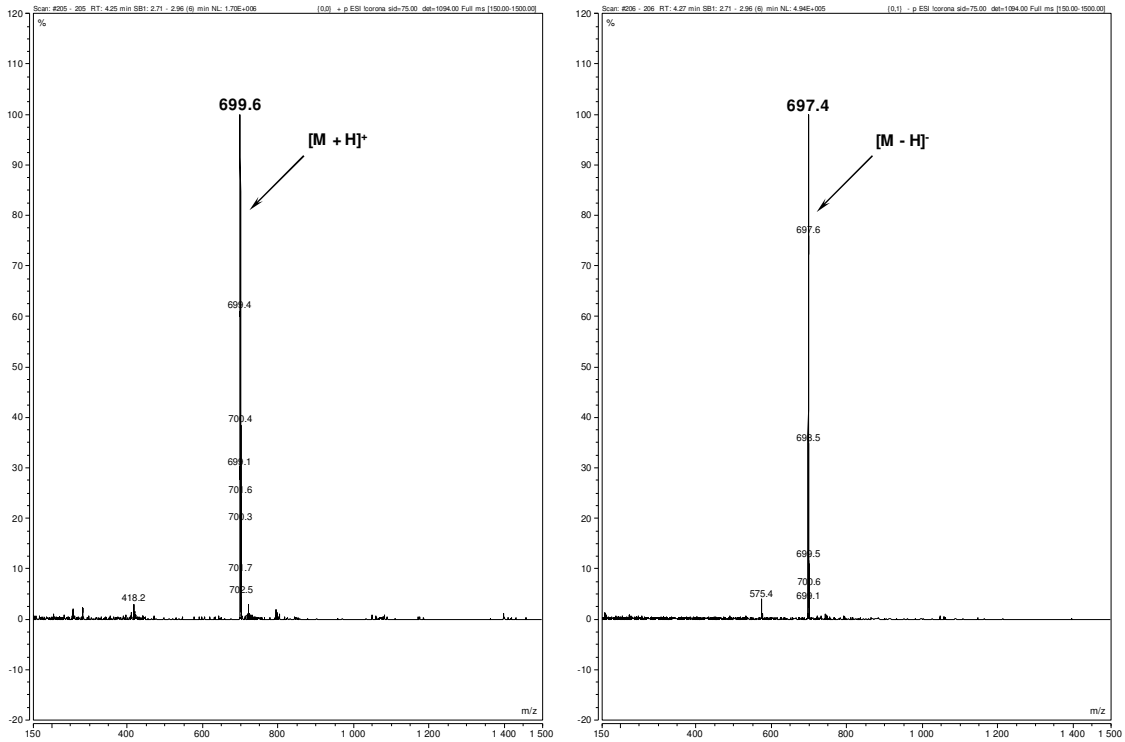


^{19}F NMR (282 MHz, [D_6]DMSO)

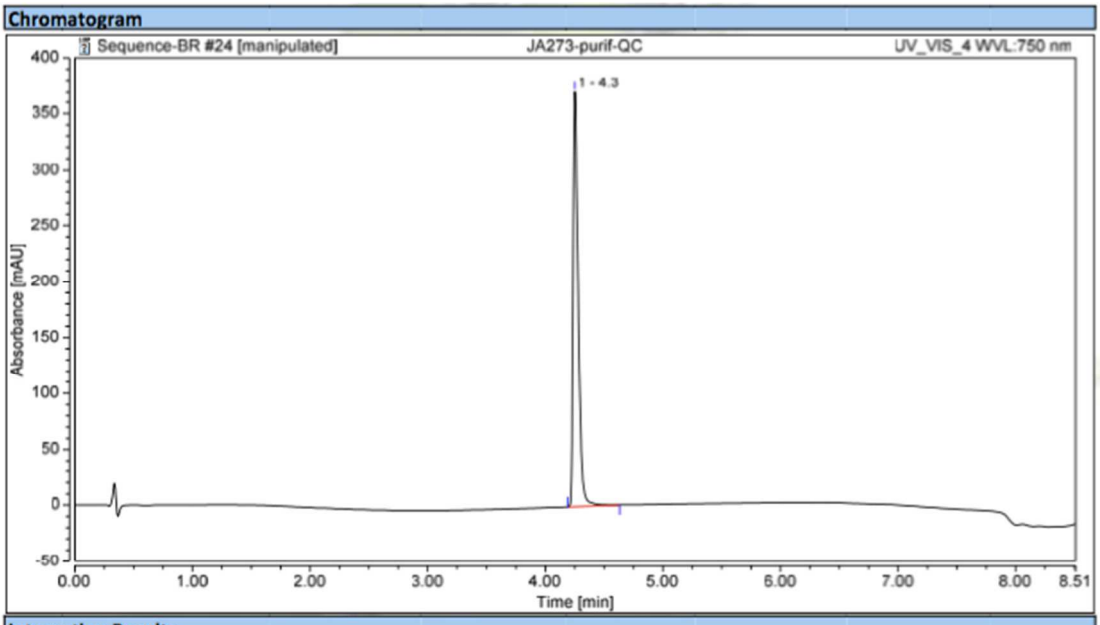
ESI HR mass spectrum (positive mode)



ESI LR mass spectra (positive & negative mode)



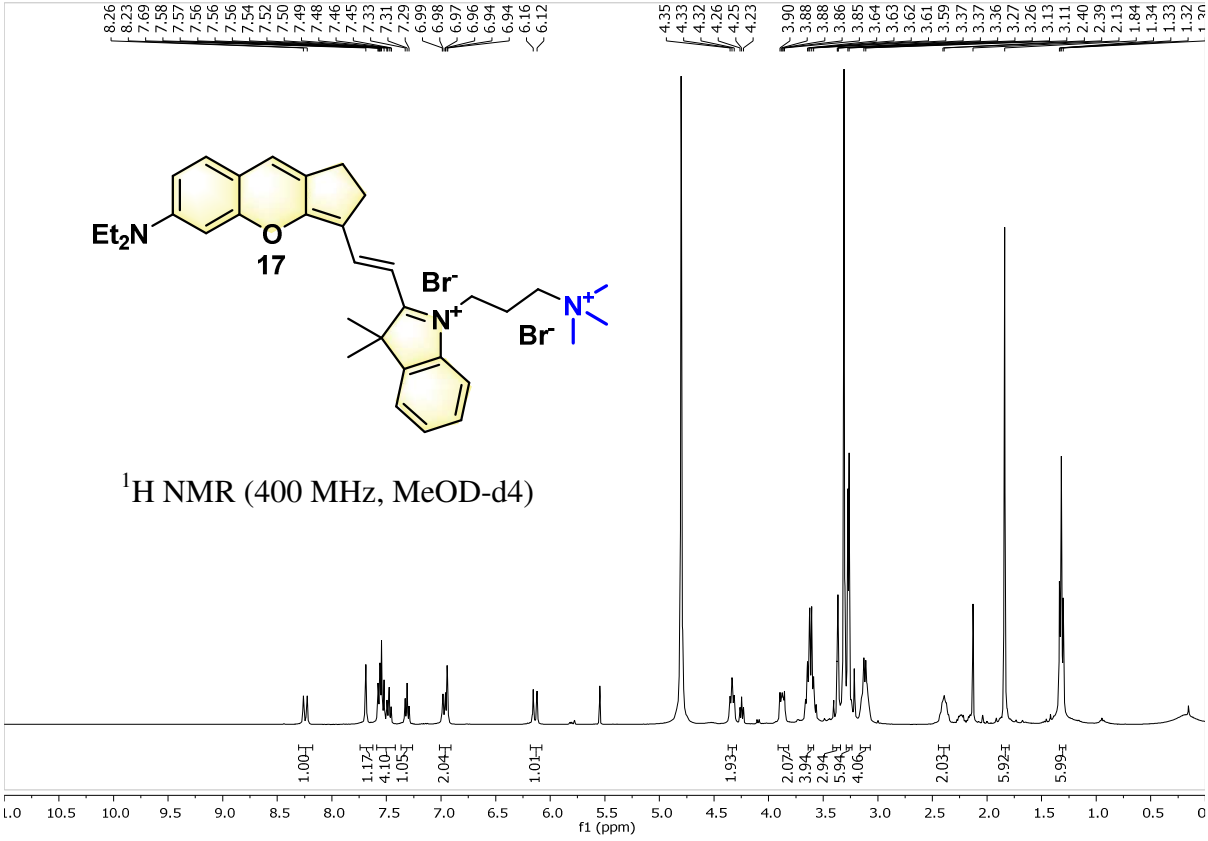
RP-HPLC elution profile (system A, detection at 750 nm)

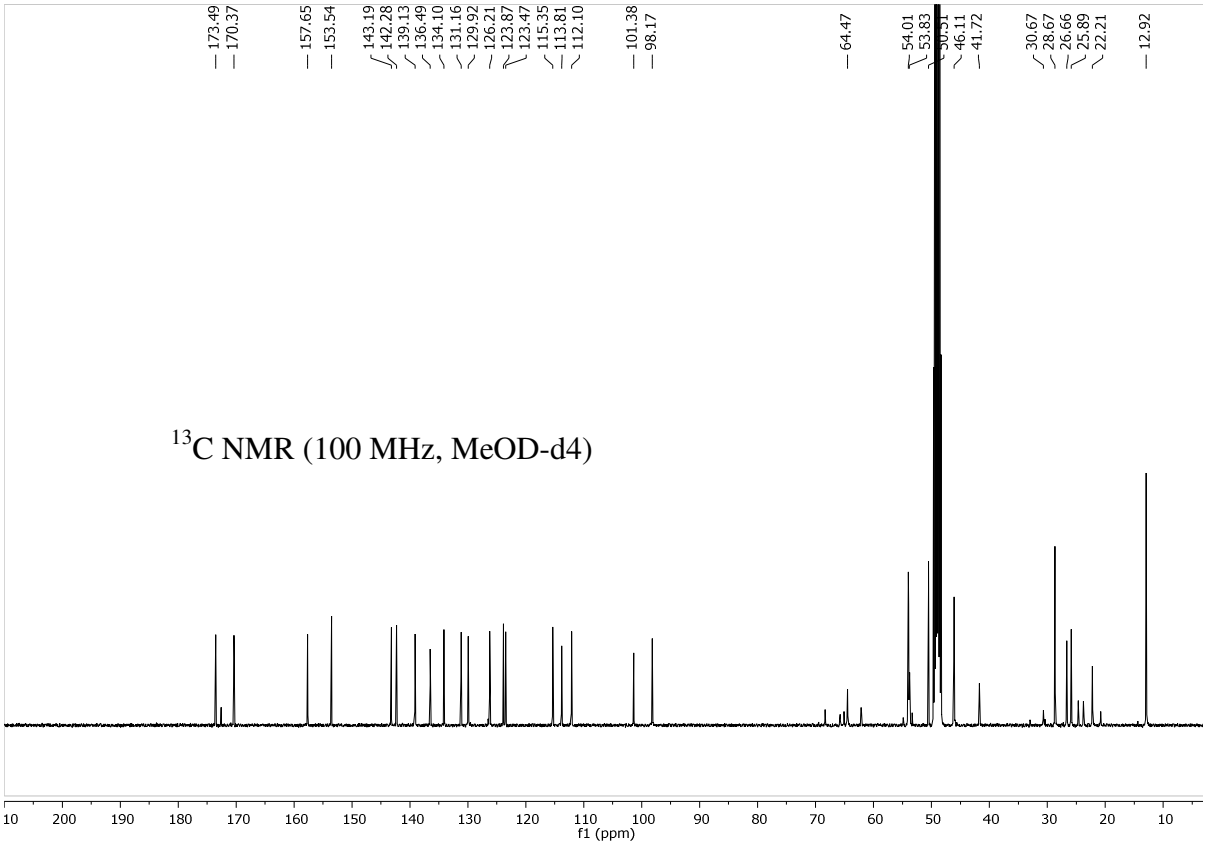


Integration Results

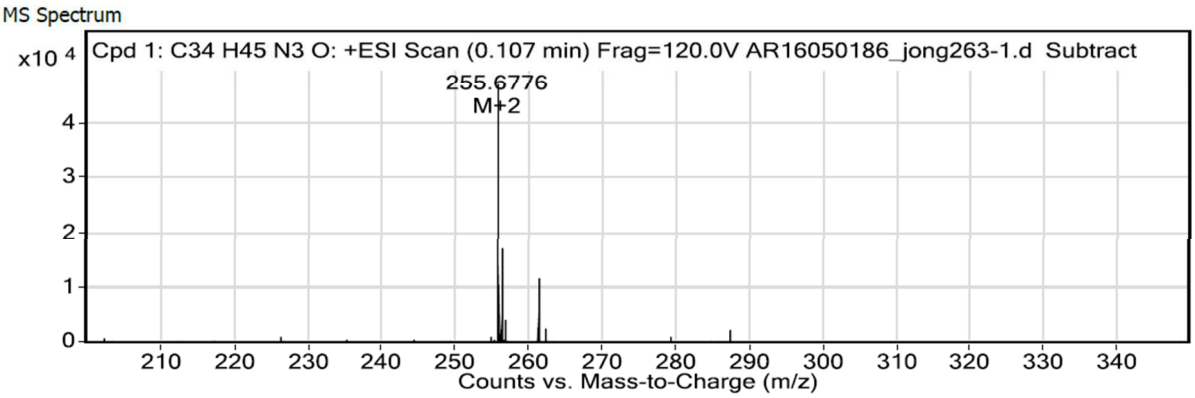
No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		4.250	18.832	371.827	100.00	100.00	n.a.
Total:			**18.832**	**371.827**	**100.00**	**100.00**	

Fluorophore 17

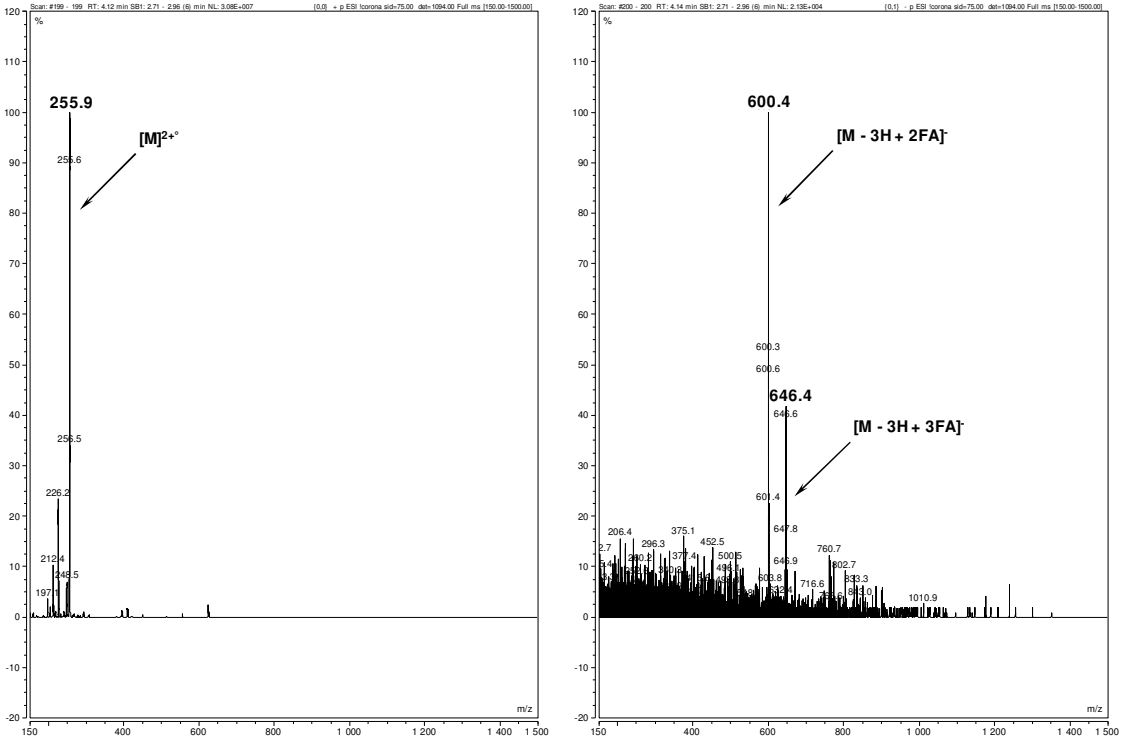




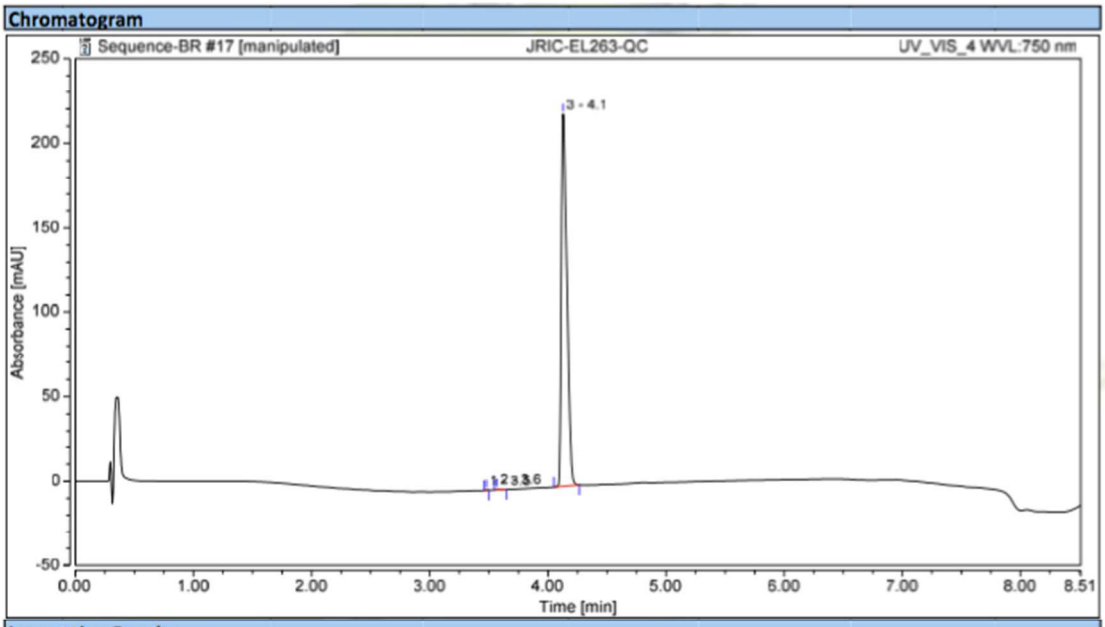
ESI HR mass spectrum (positive mode)



ESI LR mass spectra (positive & negative mode)



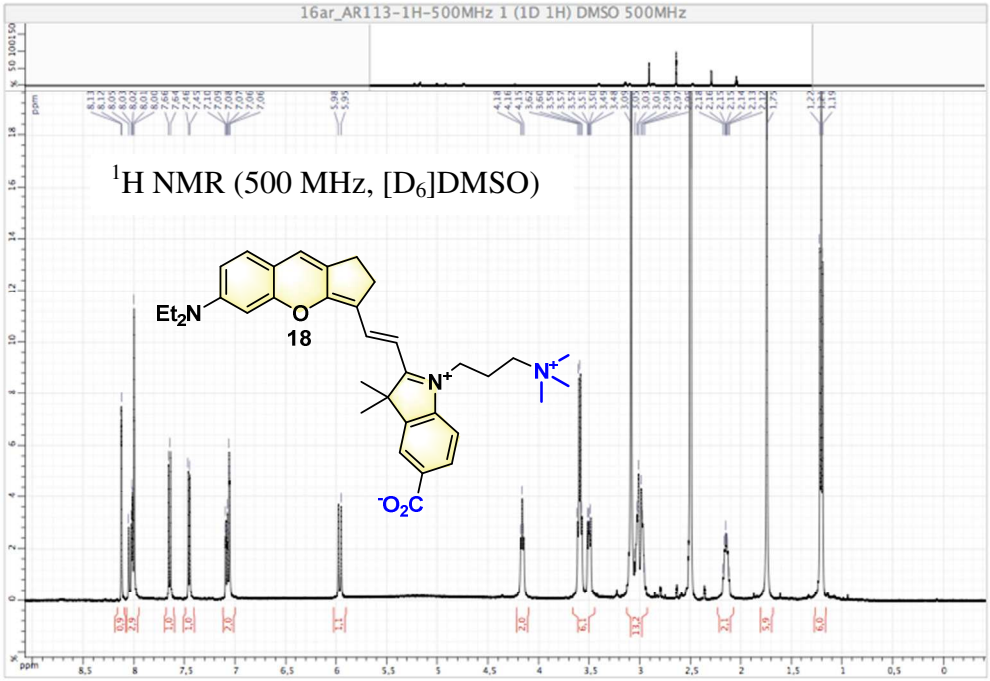
RP-HPLC elution profile (system A, detection at 750 nm)

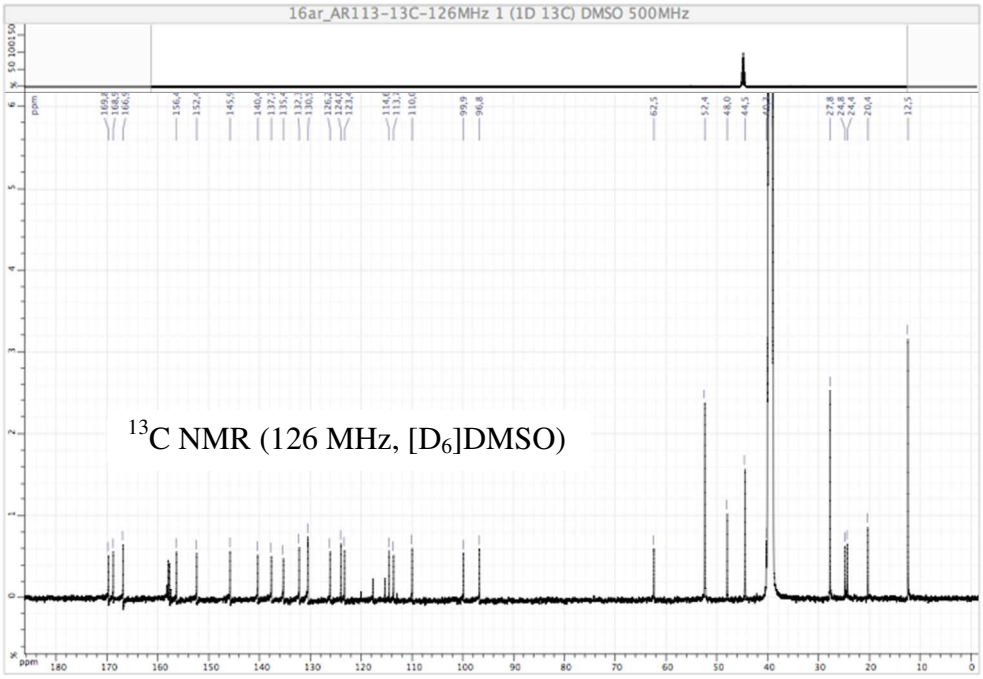


Integration Results

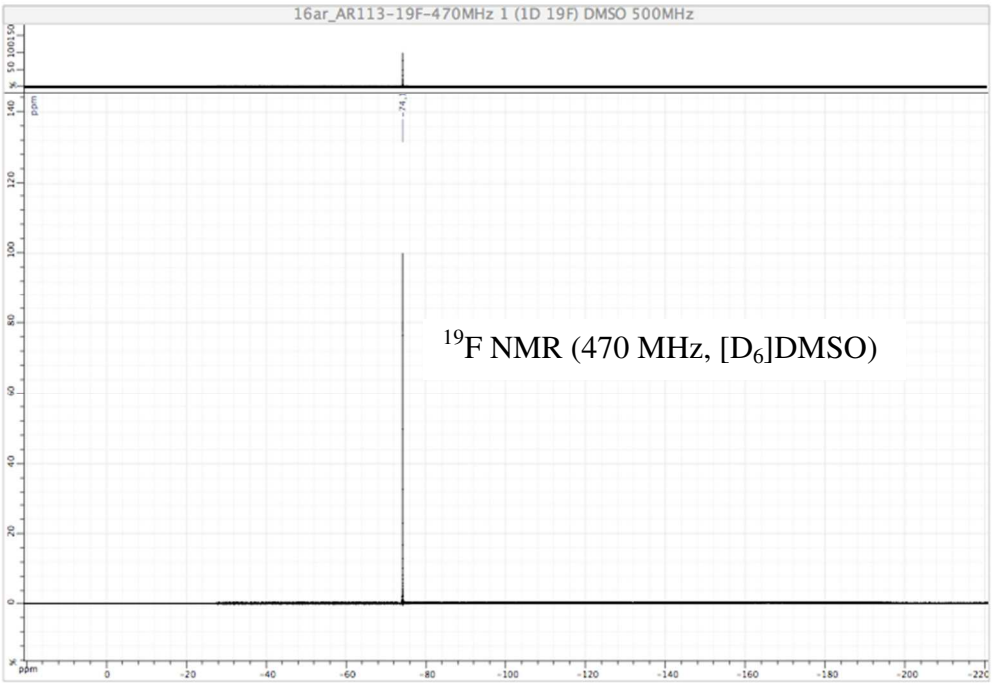
No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		3.480	0.005	0.230	0.04	0.10	n.a.
2		3.570	0.014	0.617	0.11	0.28	n.a.
3		4.130	11.992	219.999	99.84	99.62	n.a.
Total:			**12.011**	**220.846**	**100.00**	**100.00**	

Fluorophore 18



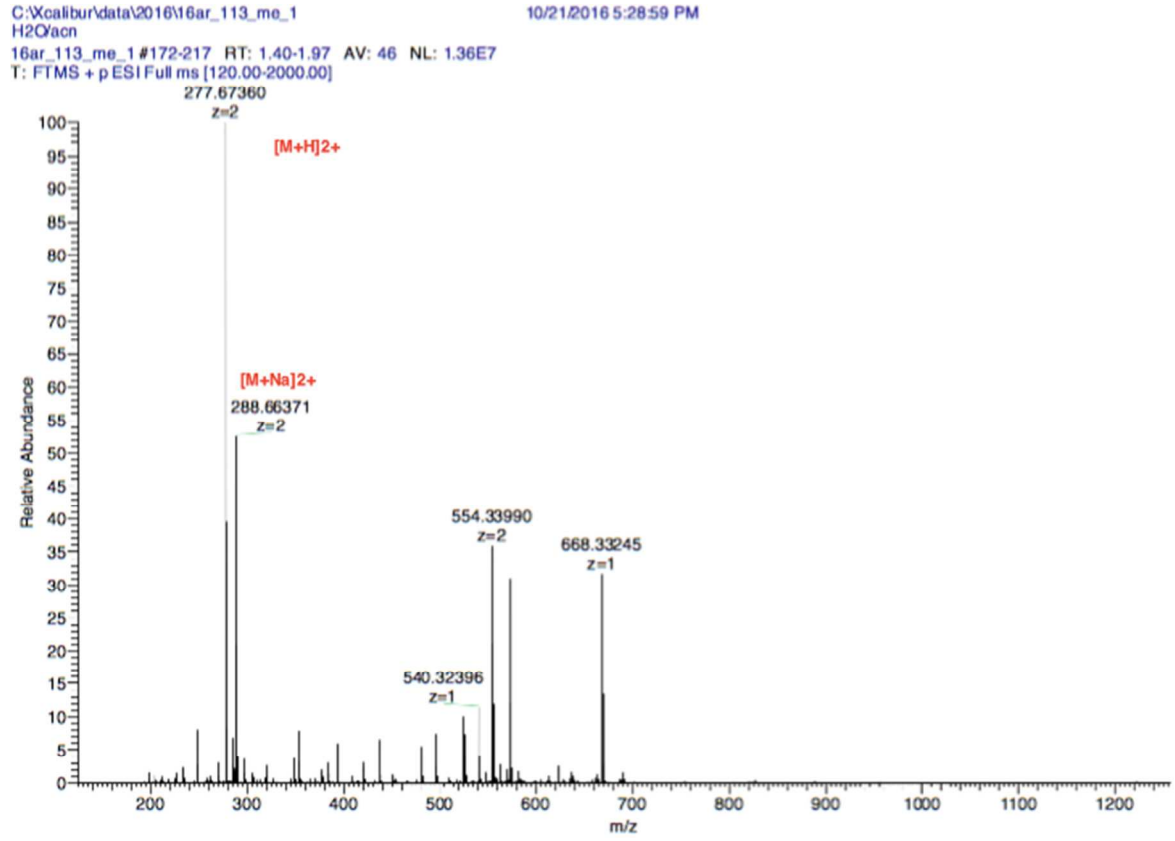


^{13}C NMR (126 MHz, [D$_6$]DMSO)

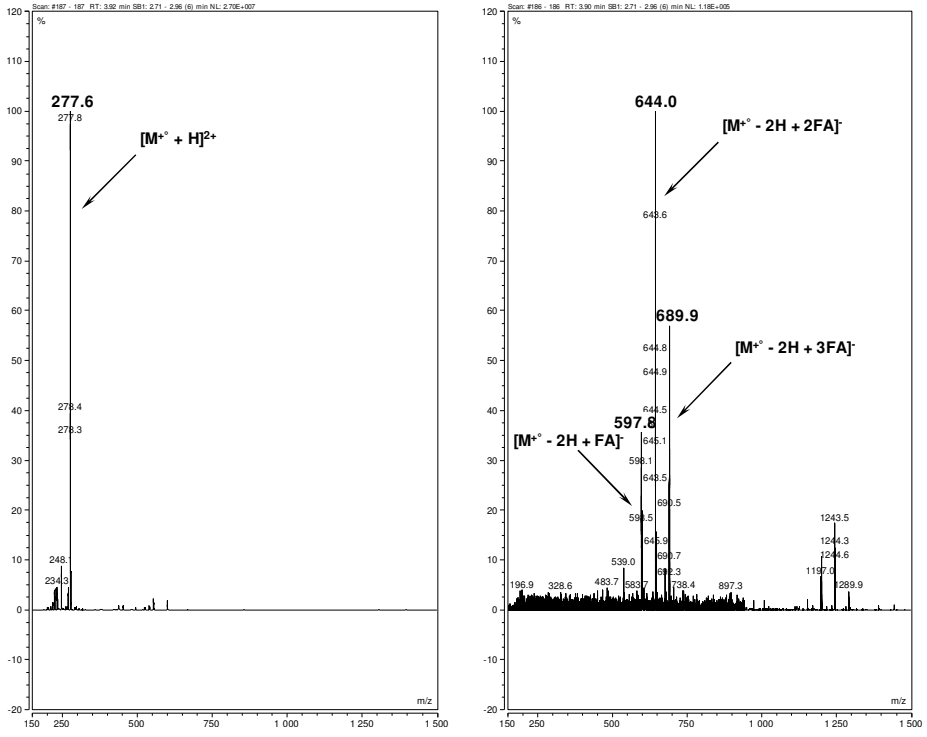


^{19}F NMR (470 MHz, [D$_6$]DMSO)

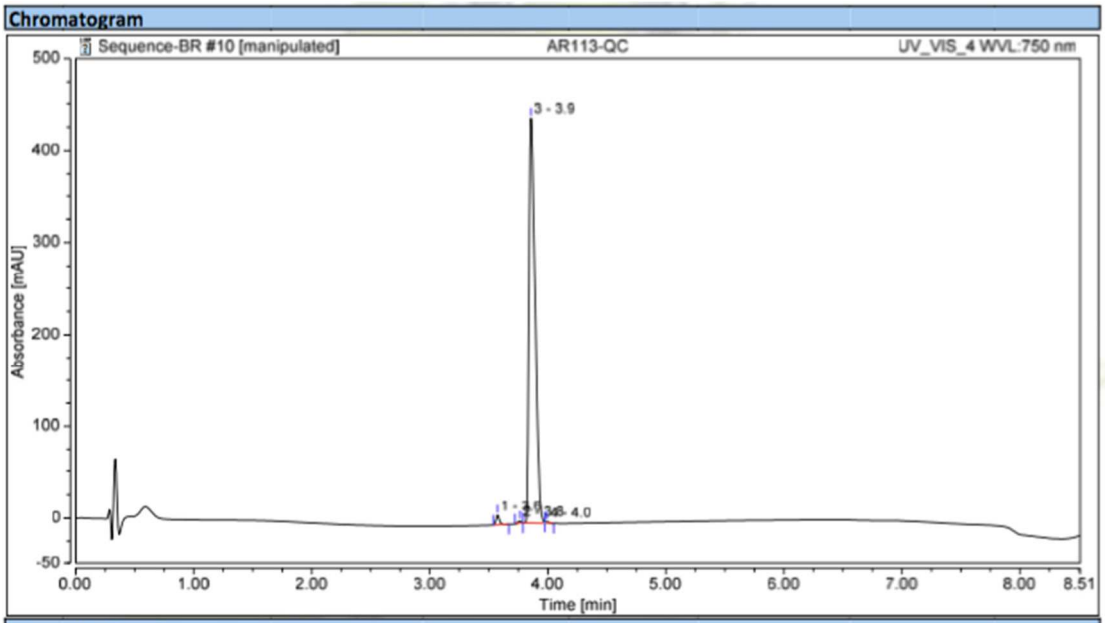
ESI HR mass spectrum (positive mode)



ESI LR mass spectra (positive & negative mode)



RP-HPLC elution profile (system A, detection at 750 nm)



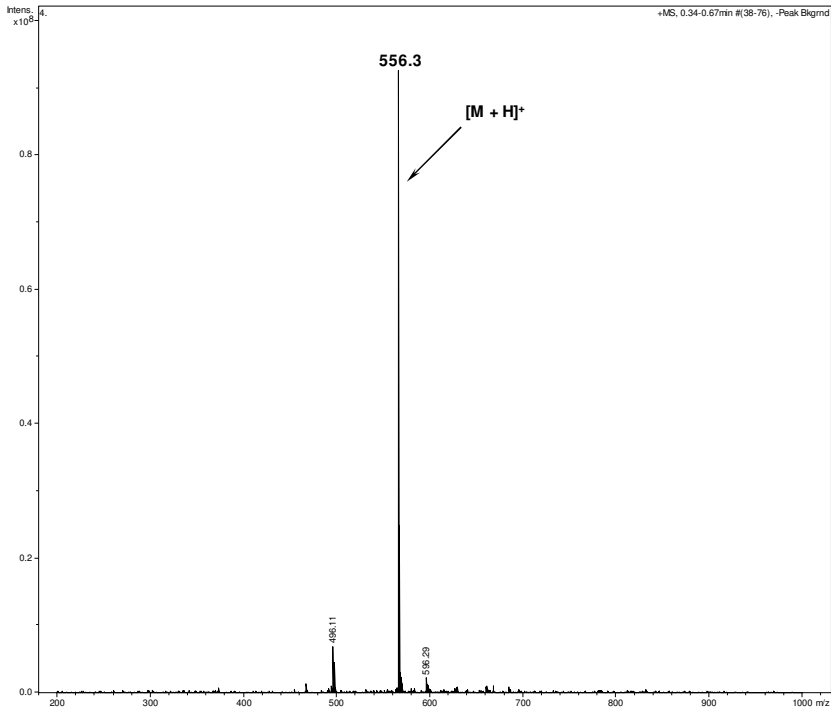
Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		3.573	0.335	10.561	1.24	2.32	n.a.
2		3.763	0.078	2.451	0.29	0.54	n.a.
3		3.857	26.412	439.975	98.23	96.79	n.a.
4		3.993	0.063	1.595	0.23	0.35	n.a.
Total:			**26.887**	**454.582**	**100.00**	**100.00**	

3) Analytical data related to the preparation and characterization of fluorescent BSA conjugates

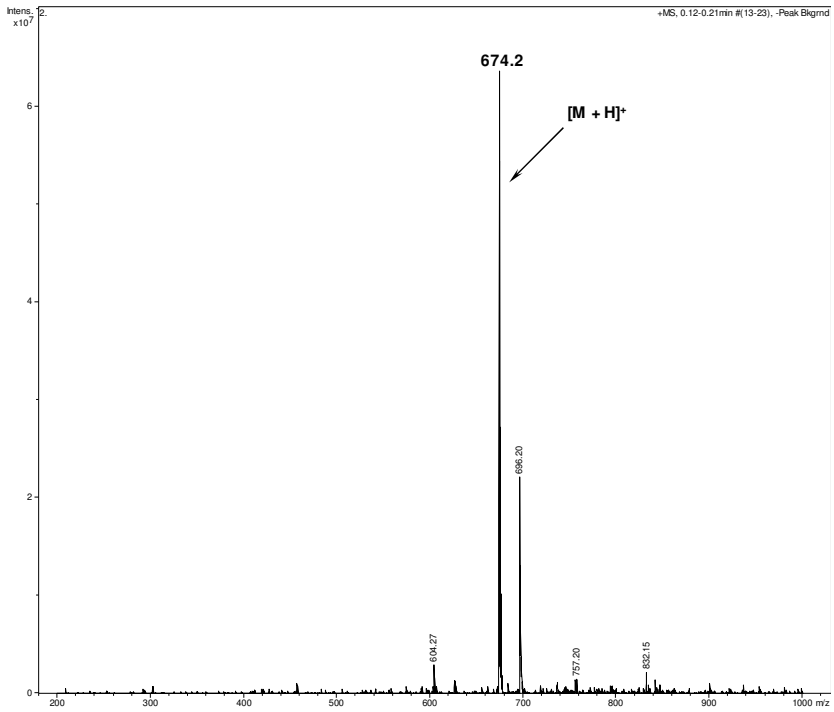
NHS ester of nor-DHX-hemicyanine fused NIR dye 12

ESI LR mass spectrum (positive mode)



NHS ester of *nor*-DHX-hemicyanine fused NIR dye 15

ESI LR mass spectrum (positive mode)



NHS ester of *nor*-DHX-hemicyanine fused NIR dye 18

ESI LR mass spectrum (positive mode)

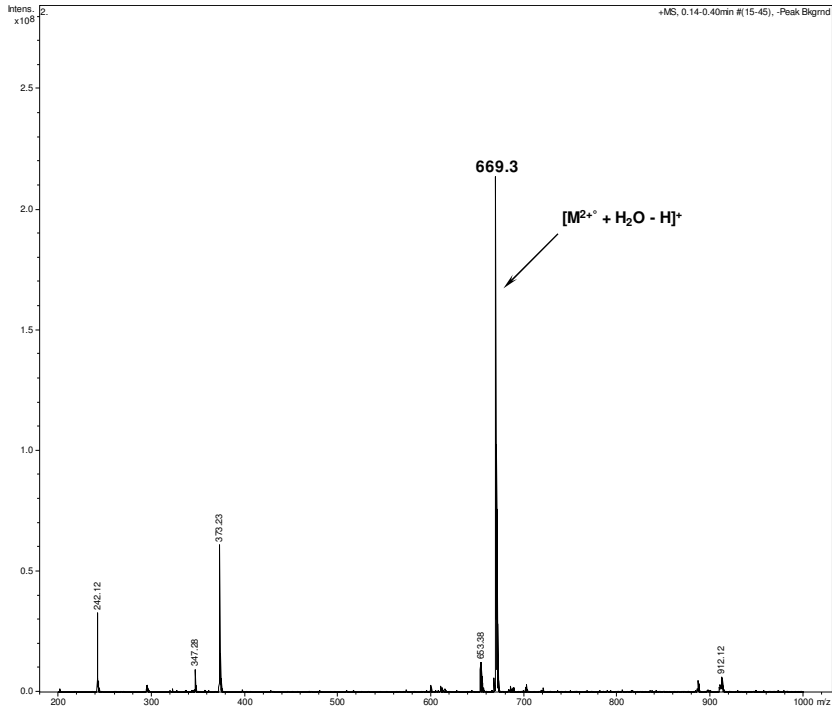


Figure S1. MALDI-TOF mass spectra of fluorescent conjugates BSA-12 (a), BSA-15 (b) and BSA-18 (c) (30 eq., pH 7.0)

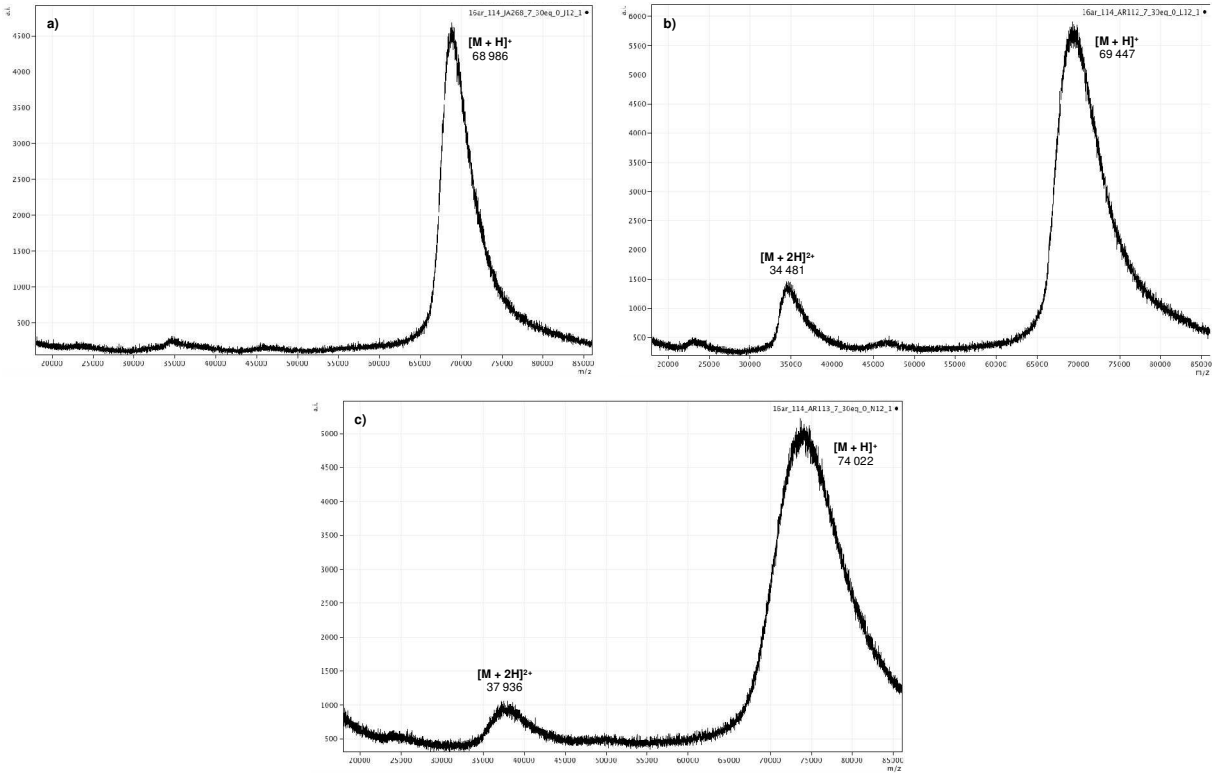


Figure S2. MALDI-TOF mass spectra of fluorescent conjugates BSA-12 (a), BSA-15 (b) and BSA-18 (c) (15 eq., pH 7.7)

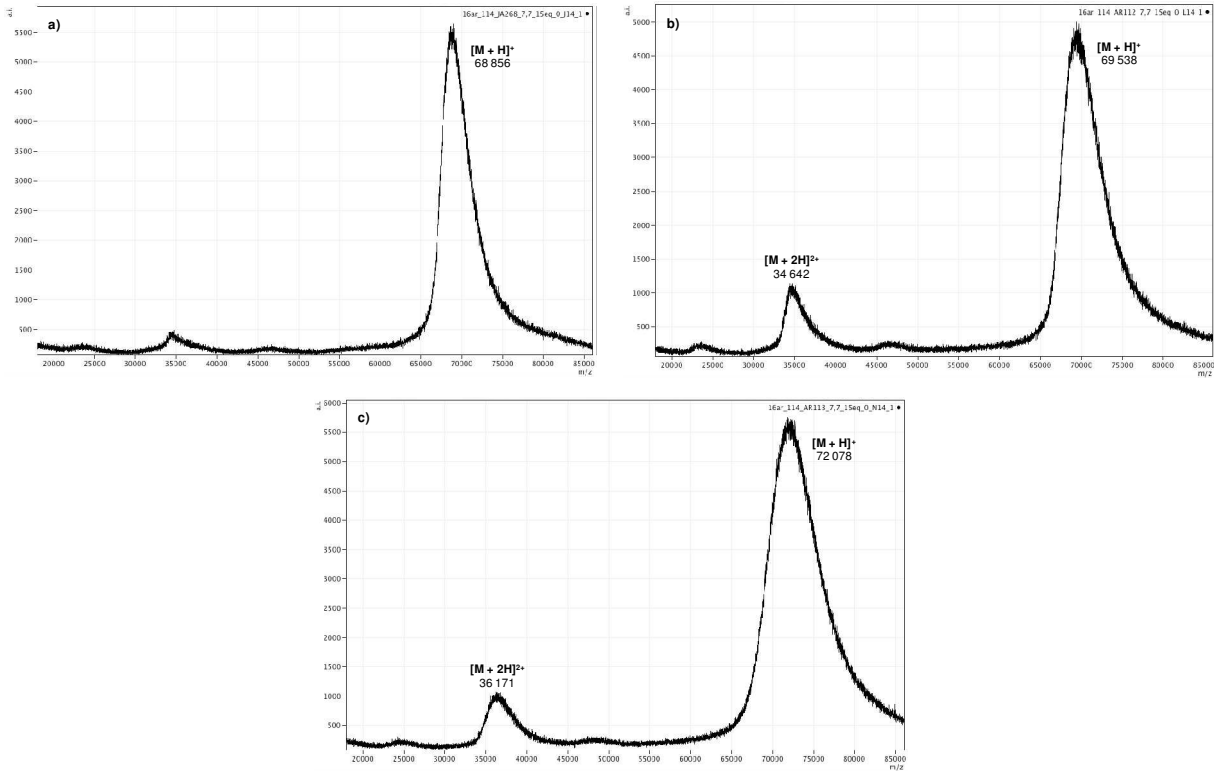


Figure S3. MALDI-TOF mass spectra of fluorescent conjugates BSA-12 (a), BSA-15 (b) and BSA-18 (c) (30 eq., pH 7.7)

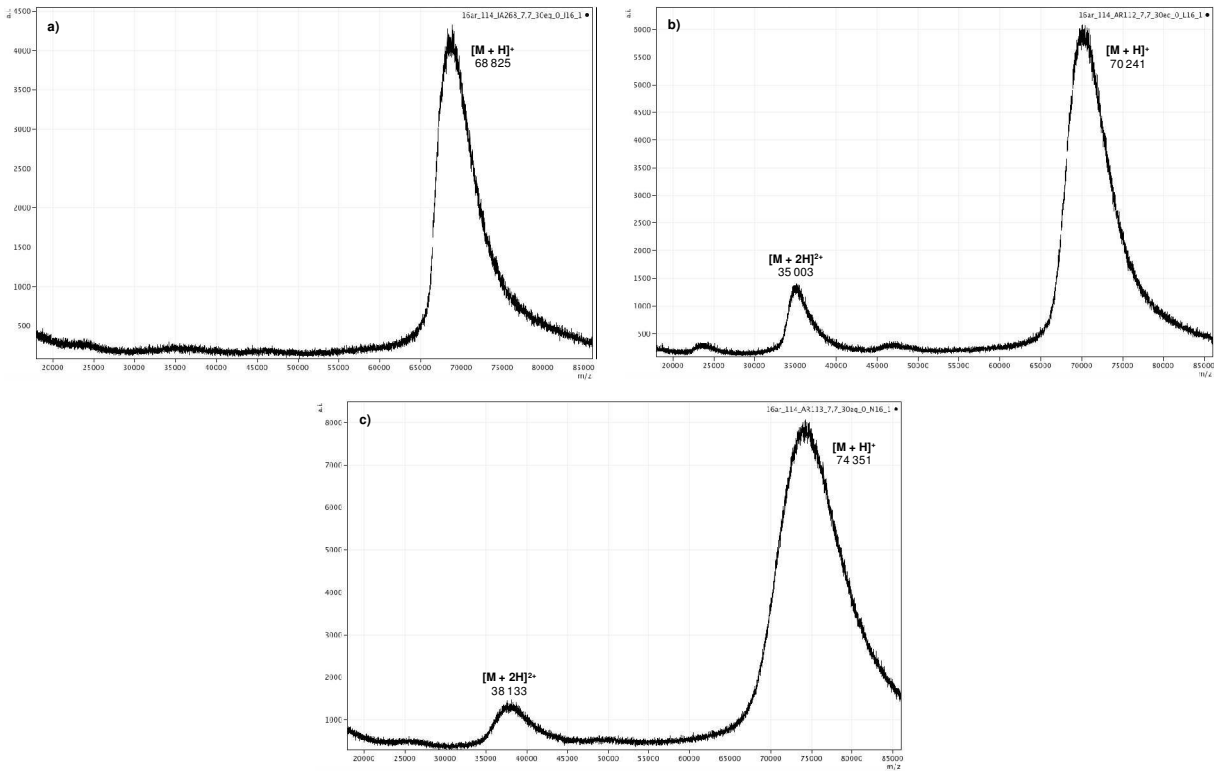


Figure S4. Gel image of fluorescent conjugates BSA-12, BSA-15 and BSA-18 prepared in phosphate buffer (pH 7.0), fluorescence scan at 790 nm upon excitation at 660 nm

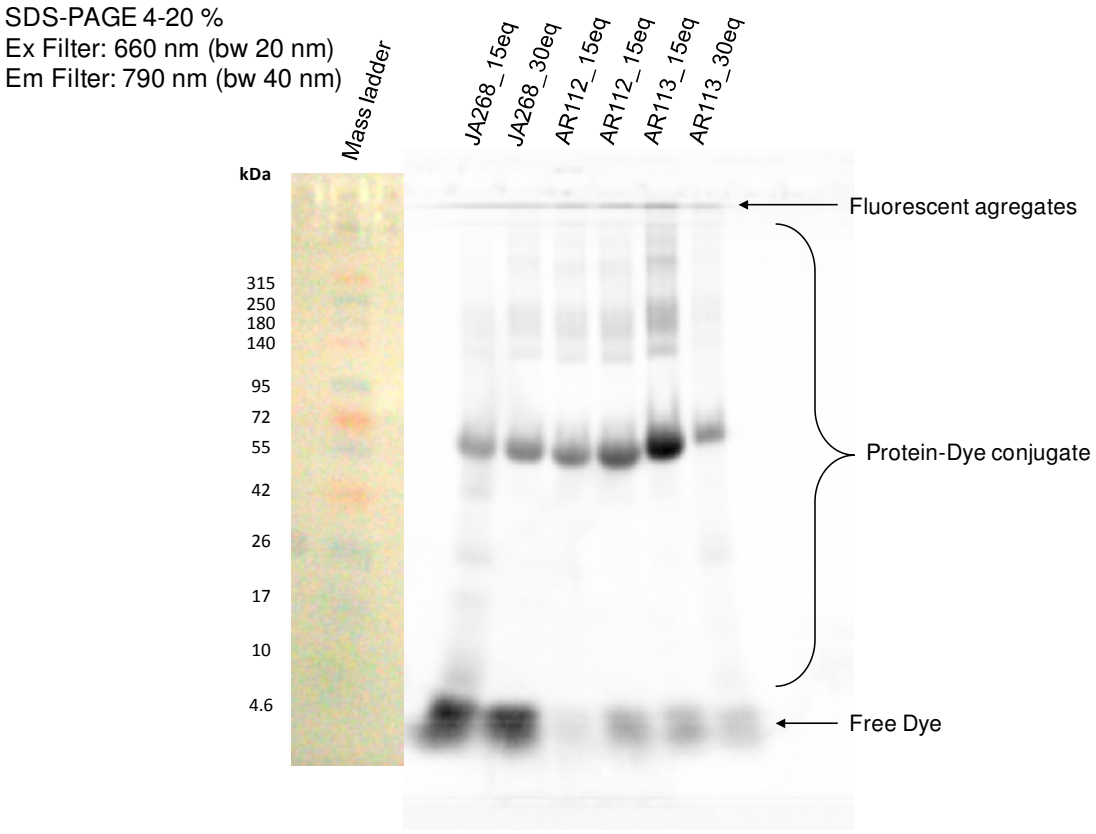
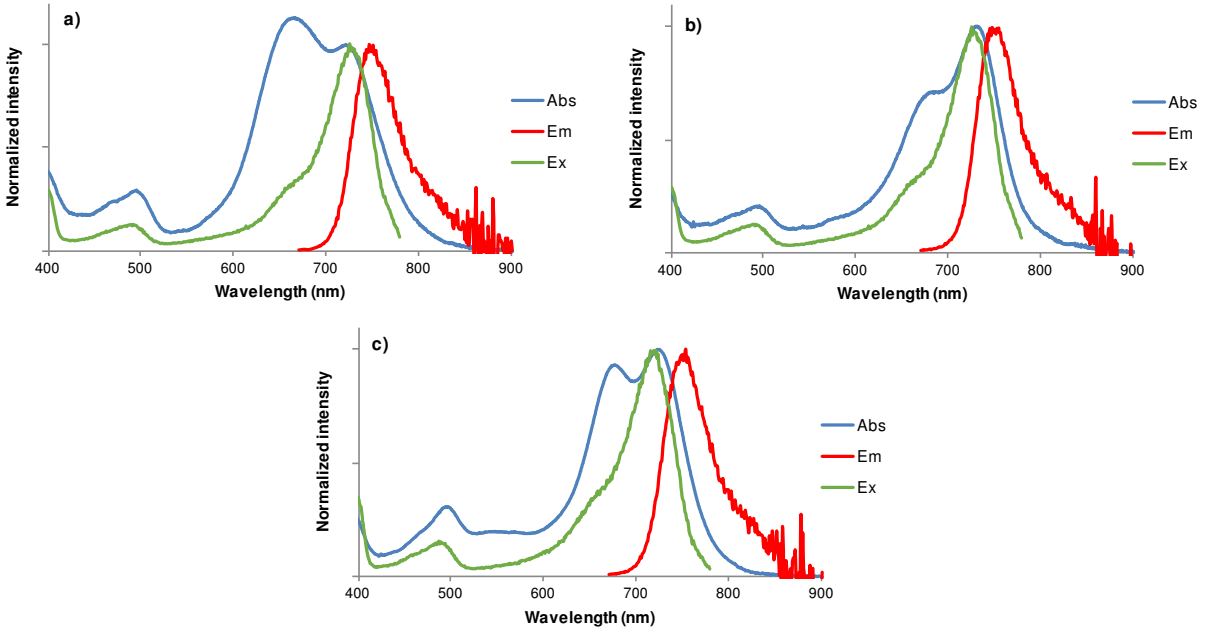


Figure S5. Absorption, excitation (Em = 800 nm) and emission (Ex = 650 nm) spectra of fluorescent conjugates BSA-12, BSA-15 and BSA-18 at 25 °C (prepared in phosphate buffer pH 7.0 with 15 eq. of NHS ester)

Please note: for all absorption spectra, the main absorption band is assigned to S0→S1 transition. The second one (far less intense) located in the range 450-500 nm is assigned to S0→S2 transition. All emission spectra (also those of standard namely ICG) are corrected until 850 nm, which explains the artefact observed at this wavelength.

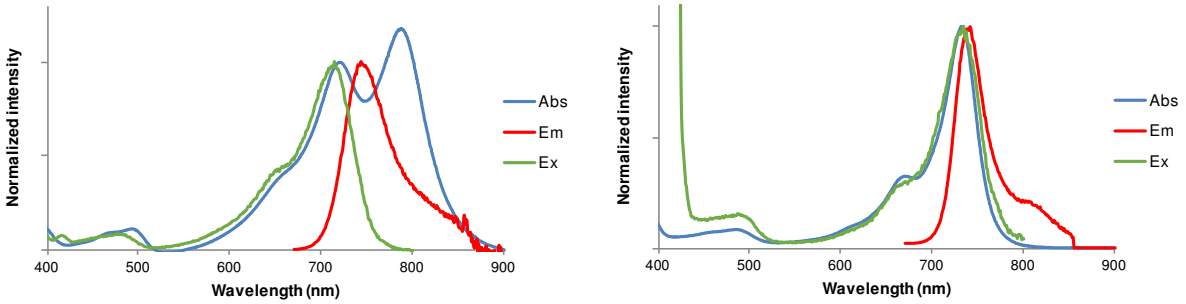


4) Absorption, excitation and emission spectra of water-soluble *nor*-DHX-hemicyanine fused NIR dyes

Please note: for all absorption spectra, the main absorption band is assigned to S0→S1 transition. The second one (far less intense) located in the range 450-500 nm is assigned to S0→S2 transition. For some excitation spectra, peak at 410/415 nm (λ_{ex}/2) assigned to Rayleigh scattering is observed. All emission spectra (also those of standard namely ICG) are corrected until 850 nm, which explains the artefact observed at this wavelength. UV-vis spectra recorded in the range 10^{-6} - 10^{-5} M for determination of molar extinction coefficients. Fluorescence Ex/Em spectra recorded in the range 10^{-7} - 10^{-6} M for QY determinations.

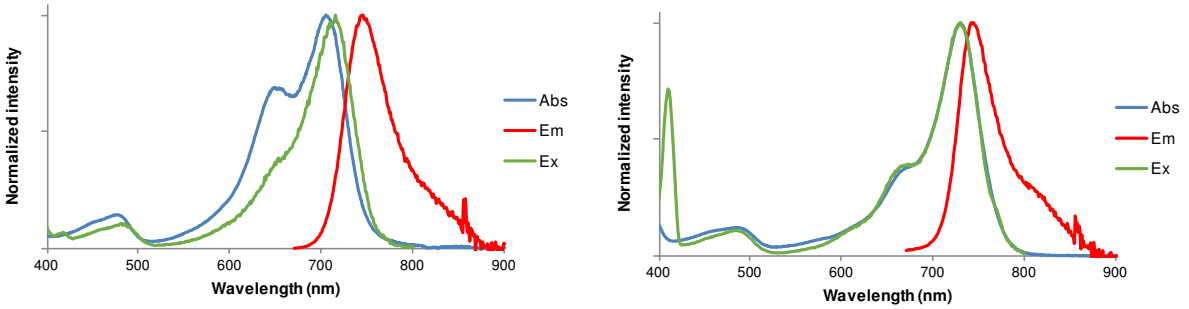
Fluorophore 8

PBS (left) & PBS + 5% BSA (right), 25 °C excitation at 650 nm, emission at 820 nm.



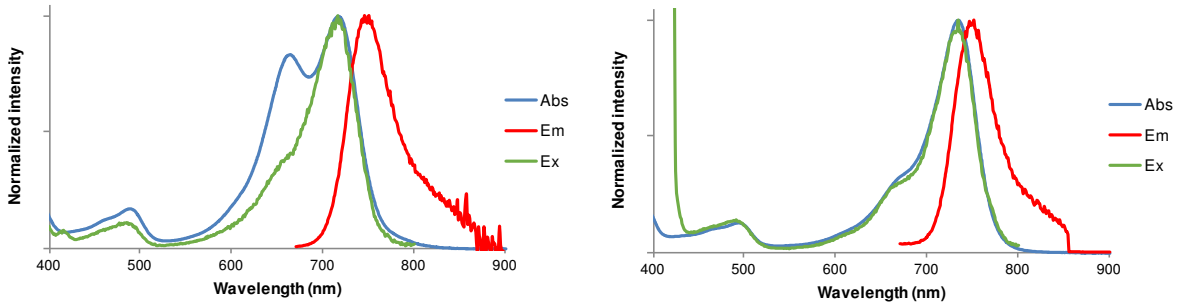
Fluorophore 9

PBS (left) & PBS + 5% BSA (right), 25 °C excitation at 650 nm, emission at 820 nm.



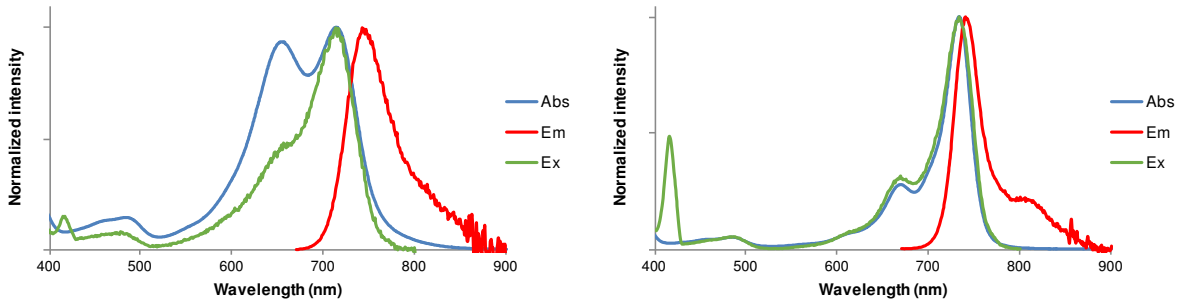
Fluorophore 10

PBS (left) & PBS + 5% BSA (right), 25 °C excitation at 650 nm, emission at 820 nm.



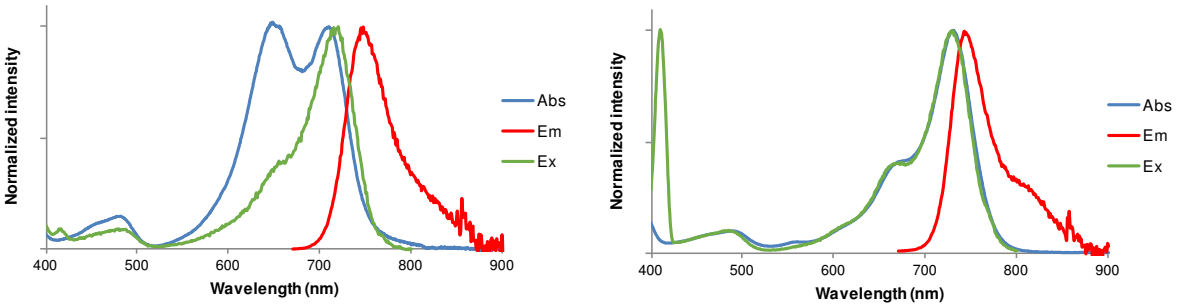
Fluorophore 11

PBS (left) & PBS + 5% BSA (right), 25 °C excitation at 650 nm, emission at 820 nm (PBS) or 830 nm (PBS + 5% BSA).



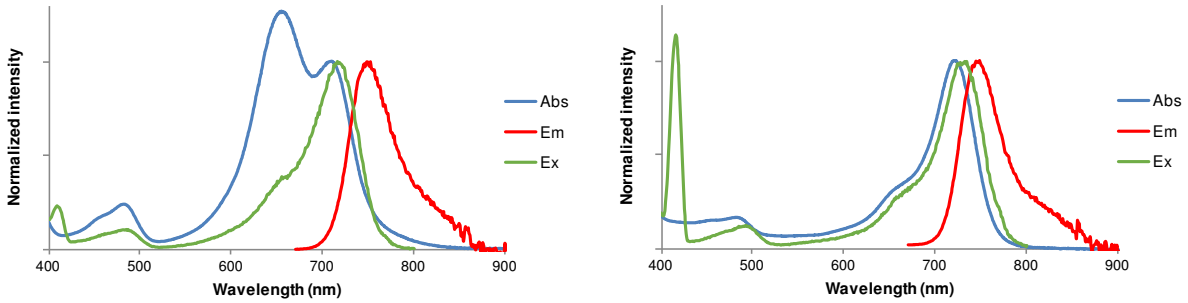
Fluorophore 12

PBS (left) & PBS + 5% BSA (right), 25 °C excitation at 650 nm, emission at 820 nm.



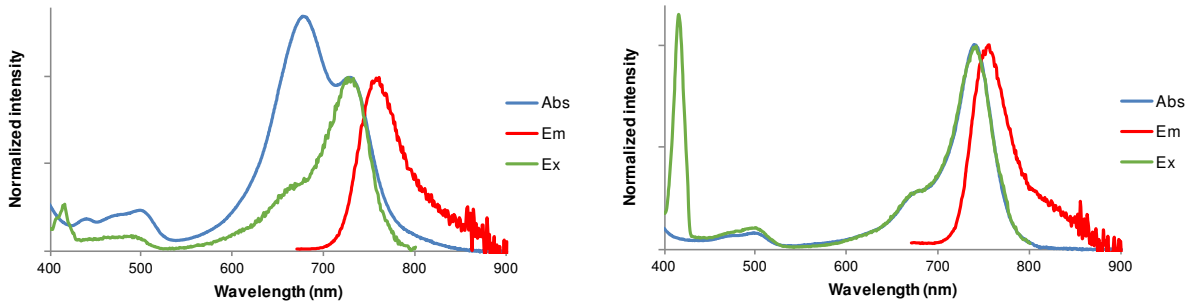
Fluorophore 13

PBS (left) & PBS + 5% BSA (right), 25 °C excitation at 650 nm, emission at 820 nm.



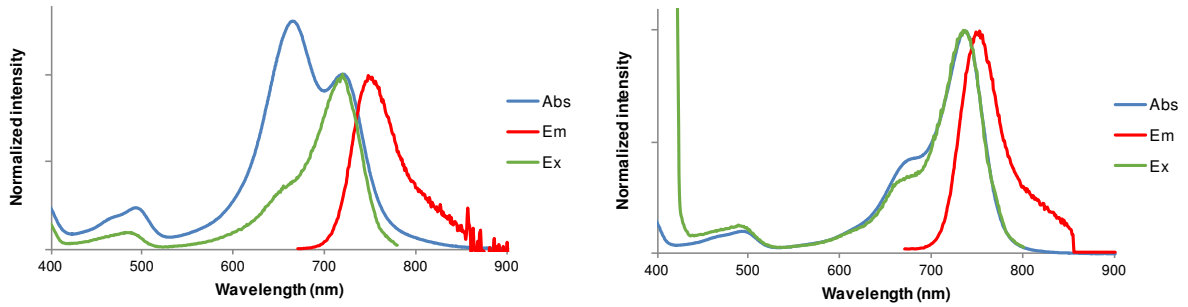
Fluorophore 14

PBS (left) & PBS + 5% BSA (right), 25 °C excitation at 650 nm, emission at 830 nm.



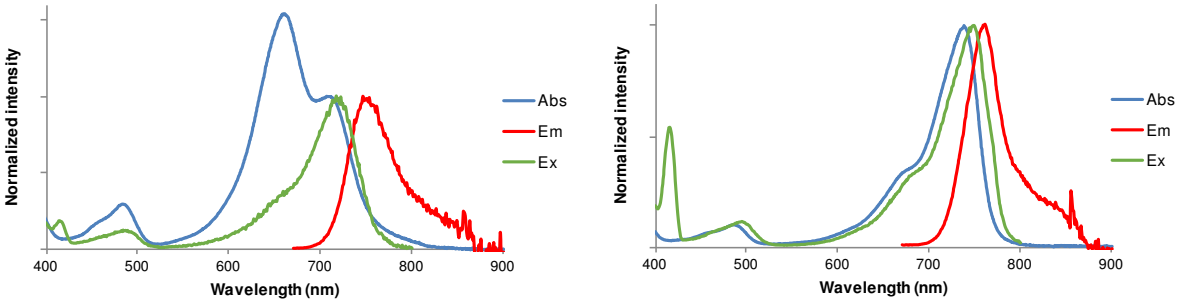
Fluorophore 15

PBS (left) & PBS + 5% BSA (right), 25 °C excitation at 650 nm, emission at 800 nm (PBS) or 820 nm (PBS + 5% BSA).



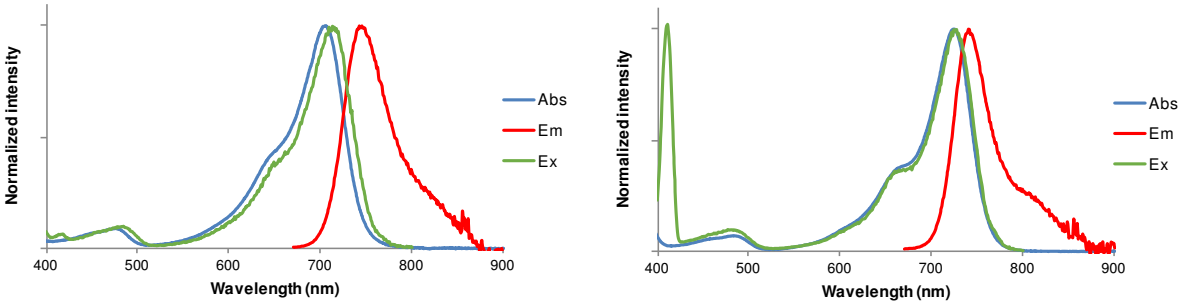
Fluorophore 16

PBS (left) & PBS + 5% BSA (right), 25 °C excitation at 650 nm, emission at 820 nm.



Fluorophore 17

PBS (left) & PBS + 5% BSA (right), 25 °C excitation at 650 nm, emission at 820 nm.



Fluorophore 18

PBS (left) & PBS + 5% BSA (right), 25 °C excitation at 650 nm, emission at 800 nm (PBS) or 820 nm (PBS + 5% BSA).