

A comparative study of the self-immolation of *para*-aminobenzylalcohol and hemithioaminal-based linkers in the context of protease-sensitive fluorogenic probes

Yves Meyer,^{a, b} Jean-Alexandre Richard,^{a, b} Bruno Delest,^b Pauline Noack,^b Pierre-Yves Renard,^{*a, b, d} and Anthony Romieu^{*a, b, c}

^a*Equipe de Chimie Bio-Organique, COBRA-CNRS UMR 6014 & FR 3038, rue Lucien Tesnière, 76131 Mont-Saint-Aignan, France, Fax: +33 2-35-52-29-71; Tel: +33 2-35-52-24-14 (or 24-15); E-mail: pierre-yves.renard@univ-rouen.fr or anthony.romieu@univ-rouen.fr*

^b*QUIDD, Technopôle du Madrillet, 50, rue Ettore Bugatti, 76800 Saint-Etienne du Rouvray, France*

^c*Université de Rouen, Place Emile Blondel, 76821 Mont-Saint-Aignan, France*

^d*Institut Universitaire de France, 103 Boulevard Saint-Michel, 75005, Paris, France*

Supporting Information

Experimental Section	S2
¹ H and ¹³ C NMR spectra of PGA pro-fluorophore VIII recorded in CDCl ₃	S13
¹ H and ¹³ C NMR spectra of PGA pro-fluorophore 3 recorded in CDCl ₃	S14
ESI-MS spectra of PGA pro-fluorophore 3 recorded in the positive (A) and negative (B) modes	S15
RP-HPLC elution profile of PGA pro-fluorophore 3 (system A)	S16
Fluorescence emission time-course of PGA pro-fluorophore 3	S16
¹ H NMR spectrum of PGA pro-fluorophore 5 recorded in CDCl ₃	S17
ESI-MS spectrum of PGA pro-fluorophore 5 recorded in the positive mode	S17
RP-HPLC elution profile of PGA pro-fluorophore 5 (system A)	S18
Fluorescence emission time-course of PGA pro-fluorophore 5	S18
¹ H and ¹³ C NMR spectra of PGA pro-fluorophore 6 recorded in CDCl ₃	S19
ESI-MS spectrum of PGA pro-fluorophore 6 recorded in the positive mode	S20
RP-HPLC elution profile of PGA pro-fluorophore 6 (system A)	S20
Fluorescence emission time-course of PGA pro-fluorophore 6	S21

Experimental Section

General Methods

Chemicals and Reagents. All solvents were dried following standard procedures (CH₃CN: distillation over CaH₂, CH₂Cl₂: distillation over P₂O₅, DMF: distillation over BaO, THF: distillation over Na^o/benzophenone, toluene: distillation over Na^o). Column chromatography were performed on silica gel (40-63 μm) from Merck. TLC was carried out in Hellendahl staining jars (50×30×90 mm), using Merck DC Kieselgel 60 F-254 aluminium sheets, and visualised by employing a short wavelength UV lamp (*i.e.*, λ = 254 nm) or staining with a 3.5% (w/v) phosphomolybdic acid solution in absolute ethanol. Pyridine was distilled over KOH and stored over BaO. Triethylamine was distilled from CaH₂ and stored over BaO. *A. Faecalis* penicillin G acylase (PGA, 0.63 U/mg) was purchased from Universität Hohenheim (Institut für Lebensmitteltechnologie, Fachgebiet Biotechnologie, Prof. Dr. L. Fischer, Stuttgart). The HPLC-gradient grade acetone and CH₃CN were obtained from Acros and Fisher Scientific respectively. Phosphate buffered saline (PBS, pH 7.5) and aq. mobile-phases for HPLC were prepared using water purified with a Milli-Q system (purified to 18.2 MΩ.cm).

Instruments. ¹H and ¹³C NMR spectra were recorded on a Bruker DPX 300 spectrometer (Bruker, Wissembourg, France). Chemical shifts are expressed in parts per million (ppm) from DMSO-*d*₆ (δ_H = 2.50, δ_C = 39.52) or CDCl₃ (δ_H = 7.26, δ_C = 77.16).¹ *J* values are expressed in Hz. Analytical HPLC was performed on a Thermo Electron Surveyor instrument equipped with a PDA detector. Semi-preparative HPLC was performed on a Finnigan SpectraSYSTEM liquid chromatography system equipped with a UV-visible 2000 detector. UV-visible absorption spectra were obtained on a Varian Cary 50 scan spectrophotometer using a rectangular quartz cell (Varian, standard cell, Open Top, 10 × 10 mm, 3.5 mL). Fluorescence spectroscopic studies were performed with a Varian Cary Eclipse spectrophotometer using a semi-micro quartz fluorescence cell (Hellma, 104F-QS, 10 × 4 mm, 1400 μL). Mass spectra were obtained with a Finnigan LCQ Advantage MAX (ion trap) apparatus equipped with an electrospray source.

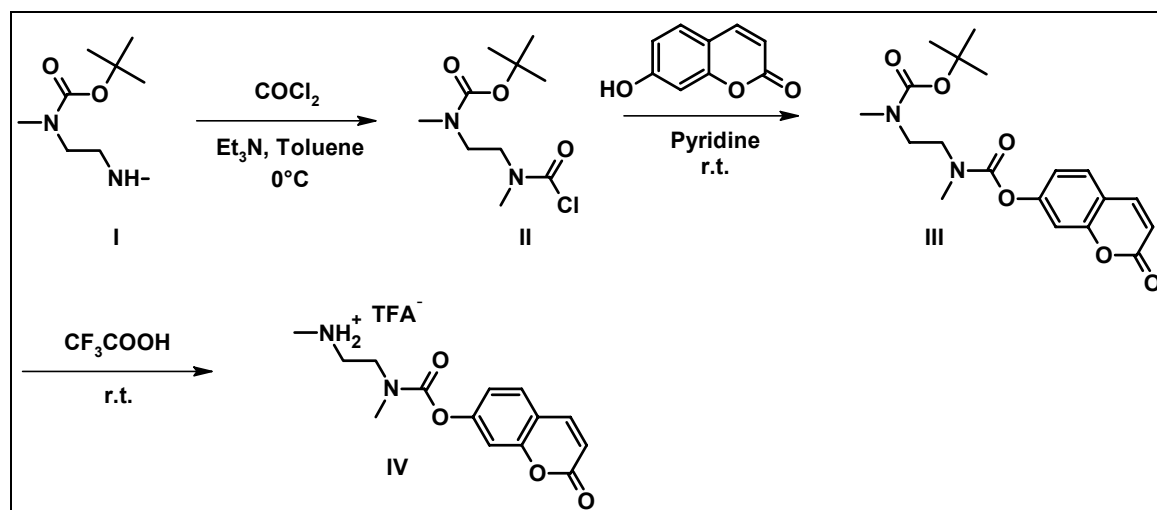
HPLC separations

Several chromatographic systems were used for the analytical experiments and the purification steps:

- **System A:** RP-HPLC (Thermo Hypersil GOLD C₁₈, 5 μm, 4.6 x 150 mm) with CH₃CN and 0.1% aq. trifluoroacetic acid (TFA 0.1%, pH 2.0) as eluents [80% TFA (5 min), then linear gradient from 20 to 90% (35 min) of CH₃CN] at a flow rate of 1.0 mL min⁻¹. UV-vis detection with the "Max Plot" (*i.e.*, chromatogram at absorbance maximum for each compound) mode (220-750 nm).

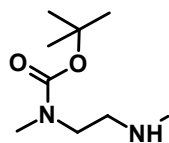
- **System B:** semi-preparative RP-HPLC (Thermo Hypersil GOLD C₁₈, 5 μm, 10 x 250 mm) with CH₃CN and 0.1% aq. trifluoroacetic acid (TFA 0.1 %, pH 2.0) as eluents [100% TFA (2 min), then linear gradient from 0 to 90 % (45 min) of CH₃CN, then linear gradient from 90 to 100 % (3 min) of CH₃CN] at a flow rate of 4 mL min⁻¹. Dual UV detection was achieved at 230 and 310 nm.

Synthetic procedures



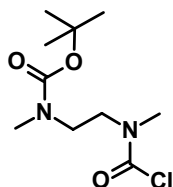
Scheme 1 Preparation of *N*-methylamino coumarin **IV**.

***tert*-Butyl methyl[2-(methylamino)ethyl]carbamate (I)**



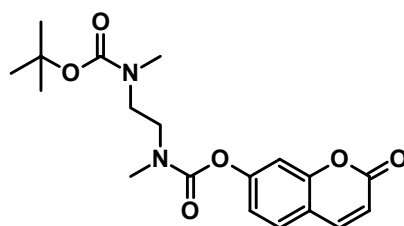
N,N'-dimethylethylenediamine (3.0 g, 34 mmol) was dissolved in dry CH₂Cl₂ (40 mL) under a N₂ atmosphere and cooled to 4 °C (ice-water bath). A solution of Boc₂O (2.4 g, 11 mmol) in dry CH₂Cl₂ (20 mL) was added dropwise then the mixture was allowed to warm at room temperature. The mixture was stirred at room temperature overnight and the solvent was removed under vacuum. The residue was extracted with EtOAc/water and the separated organic phase was washed with brine, dried over anhydrous Na₂SO₄ and evaporated under vacuum to afford a pale yellow oil (1.77 g, yield 89%). ¹H NMR (300 MHz, CDCl₃): δ 3.31 (2H, t broad, CH₂-NBoc), 2.85 (3H, s, CH₃-NBoc), 2.70 (2H, t, *J* = 6.5 Hz, CH₂-N), 2.43 (s, 3H, CH₃-N), 1.44 (s, 9H, C(CH₃)₃). All other spectroscopic data of this compound are identical to that previously reported in the literature.²

***tert*-Butyl {2-[(chlorocarbonyl)(methyl)amino]ethyl}methylcarbamate (II)**



A phosgene solution in toluene (~ 20%, 2.4 mL, 4.5 mmol) was cooled to 4 °C (ice-water bath). To this was added dropwise a mixture of compound **I** (770 mg, 4.1 mmol) and triethylamine (0.625 mL, 4.5 mmol) in dry toluene (2.5 mL). The reaction was stirred at 4 °C for 30 min then at room temperature overnight. The suspension was filtered off and the solvent removed under reduced pressure. The residue was purified by flash-chromatography on a silica gel column using a mixture of cyclohexane-EtOAc (2 : 1, v/v) as the mobile phase to afford **II** a pale yellow oil (500 mg, yield 49%) which was quickly used in the next step. *R*_f (cyclohexane-EtOAc, 2 : 1, v/v) 0.55; ¹H NMR (300 MHz, CDCl₃): δ 3.65-3.40 (m, 4H), 3.16 (s, 2H), 3.06 (s, 1H), 2.90-2.80 (m, 3H), 1.45 (s, 9H, C(CH₃)₃). All other spectroscopic data of this compound are identical to that previously reported in the literature.²

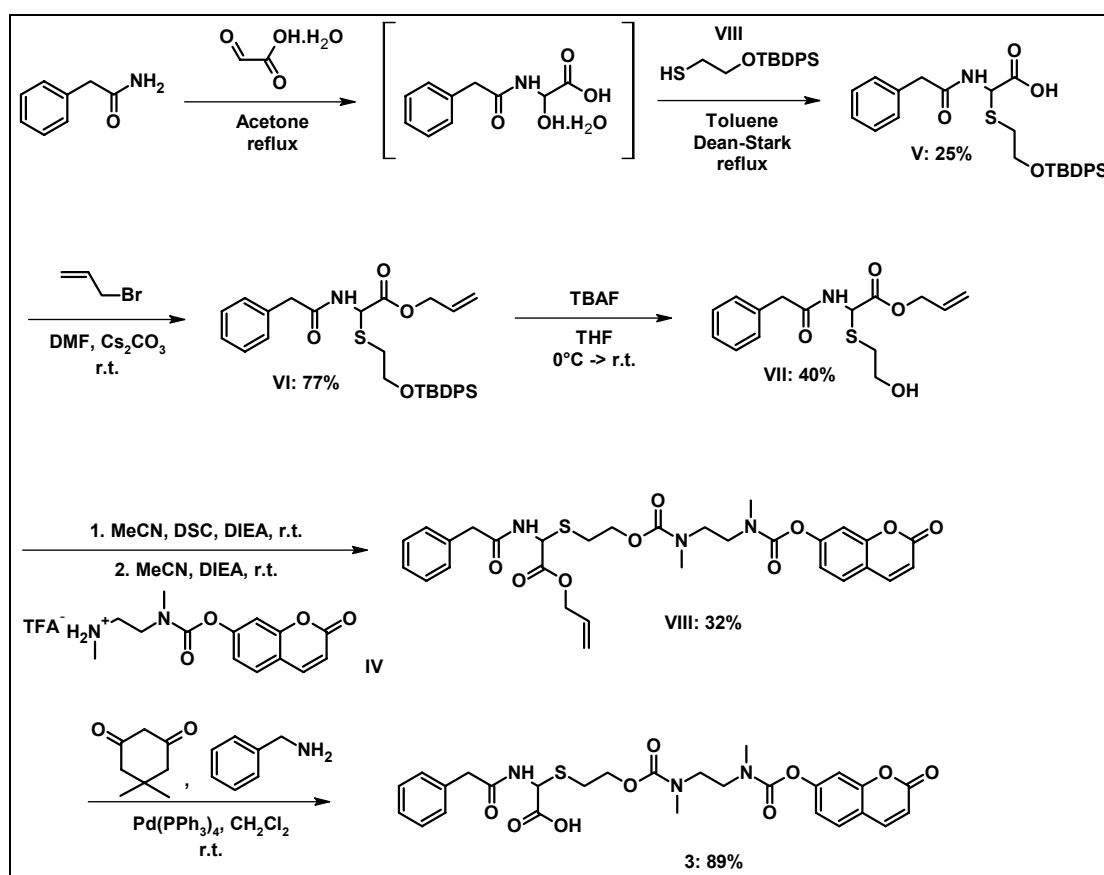
Compound (III)



7-Hydroxycoumarin (248 mg, 1.53 mmol) was added to a solution of compound **II** (500 mg, 1.99 mmol) in dry pyridine (1 mL) at room temperature under a N₂ atmosphere. The reaction was stirred for 15 h then diluted with EtOAc and washed successively with aq. 1.0 N HCl and brine. The organic layer was dried over anhydrous Na₂SO₄, filtered and evaporated to dryness. The resulting residue was purified by chromatography on a silica gel column using a mixture of cyclohexane-EtOAc (1 : 1, v/v) as the mobile phase to yield **III** as a colorless resin (500 mg, yield 87%). *R_f* (cyclohexane-EtOAc, 1 : 1, v/v) 0.21; ¹H NMR (300 MHz, CDCl₃): δ 7.68 (1H, d, *J* = 9.6 Hz, H_{coum}), 7.46 (1H, d, *J* = 8.2 Hz, H_{coum}), 7.12-7.07 (2H, m, H_{coum}), 6.36 (1H, d, *J* = 9.6 Hz, H_{coum}), 3.57-3.47 (4H, m) 3.14-3.05 (3H, m), 2.92-2.89 (3H, m), 1.44 (s, 9H, C(CH₃)₃); ¹³C NMR (75.4 MHz, CDCl₃): δ 160.6, 155.5, 154.6, 154.1, 153.9, 153.7, 143.1, 128.4, 118.8, 118.6, 116.1, 115.7, 110.6, 110.4, 110.3, 80.0, 47.4, 47.3, 47.1, 46.9, 46.4, 45.6, 35.4, 35.2, 34.6, 28.4; MS (ESI, positive mode): *m/z* 376.80 [M + H]⁺, 394.00 [M + H₂O]⁺ (water cluster formed during the mass analysis), 399.20 [M + Na]⁺, calcd mass for C₁₉H₂₄N₂O₆ 376.16.

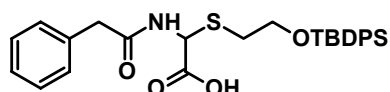
N-Methylamino coumarin (IV)

vide infra



Scheme 2 Synthesis of model pro-fluorescent compound **3**.

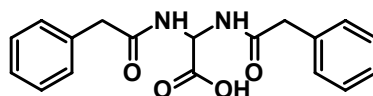
Compound (V)



Phenylacetamide (1.0 g, 7.39 mmol) and glyoxylic acid monohydrate (715 mg, 7.76 mmol) were suspended in HPLC-grade acetone (10 mL) and the mixture was refluxed for 15 h. When cooled down, non-soluble materials were filtered off and the filtrate was concentrated under reduced pressure to give a gummy solid (1.7 g). This crude material was mixed with alcohol **VIII**³ (1.25 g, 3.95 mmol) and *para*-toluenesulfonic acid (PTSA, 100 mg) in dry toluene (10 mL) and the mixture was refluxed in a Dean-Stark trap for 4 h. The solvent was evaporated and the residue was dissolved in a mixture of EtOAc and deionised water (100 mL each). Using strong stirring, the pH was adjusted to ~ 10 by slow addition of a saturated aq. K₂CO₃ solution. The organic layer was separated and the aqueous layer[¶] was extracted twice with EtOAc (2 x 100 mL). The combined organic layer was dried over anhydrous Na₂SO₄ and the solvent was removed under vacuum to give the crude **V** which was purified by chromatography on silica gel column using successively the following mobile phases:

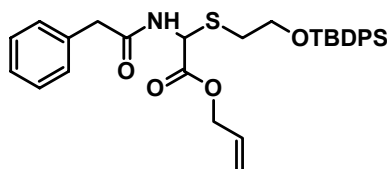
cyclohexane-EtOAc (1 : 1, v/v), 100% EtOAc 100% and CH₂Cl₂-MeOH (9 : 1, v/v) to give hemithioaminal **V** as a pale yellow amorphous solid (500 mg, yield 25%). ¹H NMR (300 MHz, CDCl₃): δ 7.70-7.45 (4H, m, H_{Ar}), 7.44-7.15 (5H, m, H_{Ar}), 7.14-6.80 (6H, m, H_{Ar}), 5.34 (1H, m, CH), 3.70-3.10 (4H, m, CH₂ + OCH₂), 2.70-2.40 (2H, m, SCH₂), 0.95 (9H, bs, CH(CH₃)₃). The complexity of the spectrum (rotamers, slow relaxation rate of some protons) for this compound makes a complete assignment impossible. MS (ESI, negative mode): *m/z* 506.13 [M - H]⁻, 1013.13 [2M - H]⁻, calcd mass for C₂₈H₃₃NO₄SSi 507.19.

¹H aq. layer was acidified to pH ~ 3.0 by slow addition of solid KHSO₄ to give a white precipitate which was removed by filtration and identified as the aminal derivative (460 mg, yield 19%):



¹H NMR (300 MHz, DMSO-*d*₆): δ 12.87 (1H, bs, COOH), 8.94 (2H, d, *J* = 8.0 Hz, 2 x NH), 7.29-7.20 (10H, m, H_{Ar}), 5.53 (1H, t, *J* = 8.0 Hz, CH), 3.48 (4H, s, 2 x CH₂); MS (ESI, negative mode): *m/z* 325.13 [M - H]⁻, calcd mass for C₁₈H₁₈N₂O₄ 326.12.

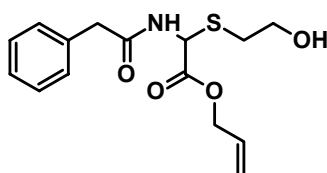
Compound (VI)



Compound **V** (100 mg, 0.197 mmol) was dissolved in dry DMF (500 μL) and allyl bromide (19 μL, 0.216 mmol) was added at room temperature under a N₂ atmosphere. The resulting mixture was cooled to 0 °C and anhydrous Cs₂CO₃ (320 mg, 0.985 mmol) was quickly added. After the addition, the reaction was stirred at room temperature for 3 h. Upon completion, the reaction was diluted with saturated aq. NaCl and EtOAc. The organic phase was separated then washed with deionised water, dried over Na₂SO₄, filtered and evaporated to dryness. The resulting residue was purified by chromatography on a silica gel column using a mixture of cyclohexane-EtOAc (8 : 2, v/v) as the mobile phase to afford the allyl ester **VI** as a white solid (82 mg, yield 77%). *R*_f 0.60 (cyclohexane-EtOAc, 2 : 1, v/v); ¹H NMR (300 MHz, CDCl₃): δ = 7.62 (4H, m, H_{Ar}), 7.40-7.15 (11H, m, H_{Ar}), 6.31 (1H, d, *J* = 8.5 Hz, NH), 5.88-5.74 (1H, m, CH=CH₂_{allyl}), 5.50 (1H, d, *J* = 8.5 Hz, CH), 5.28 (1H, d, *J* = 17.1 Hz,

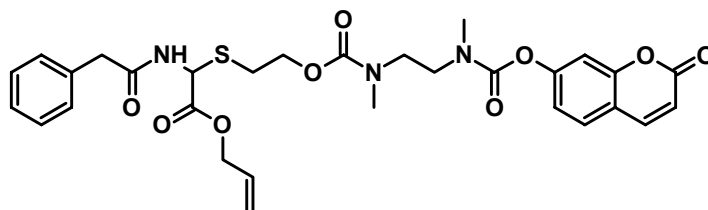
CH=CH_{2trans}), 5.19 (1H, d, $J = 10.3$ Hz, CH=CH_{2cis}), 4.63-4.51 (2H, m, OCH₂ allyl), 3.78-3.72 (2H, m, OCH₂), 3.47 (2H, s, CH₂), 2.85-2.69 (2H, m, SCH₂), 1.03 (9H, s, CH(CH₃)₃); ¹³C NMR (75.4 MHz, CDCl₃): δ 170.0, 168.7, 135.7, 134.1, 133.5, 133.4, 131.1, 129.9, 129.4, 129.1, 127.9, 127.6, 119.3, 66.6, 63.3, 53.6, 43.5, 33.4, 26.9, 19.3; MS (ESI, positive mode): m/z 548.00 [M + H]⁺, 564.80 [M + H₂O]⁺⁺ (water cluster formed during the mass analysis), calcd mass for C₃₁H₃₇NO₄SSi 547.22.

Compound (VII)



A 1.0 M solution of TBAF in THF (292 μ L, 0.292 mmol) was added to a pre-cooled (to 0 °C) solution of **VI** (80 mg, 0.146 mmol) in dry THF (430 μ L). The resulting reaction mixture was stirred under a N₂ atmosphere for 2 h. The reaction was checked for completion and volatiles were removed under reduced pressure. The resulting residue was purified by chromatography on a silica gel column using a mixture of cyclohexane-EtOAc (1 : 1, v/v) as the mobile phase to give alcohol **VII** as yellow oil (18 mg, yield 40%). R_f 0.10 (cyclohexane-EtOAc, 2 : 1, v/v); ¹H NMR (300 MHz, CDCl₃): δ 7.71 (1H, d, $J = 7.9$ Hz, NH), 7.40-7.26 (5H, m, H_{Ar}), 6.80 (1H, bd, $J = 7.5$ Hz, OH), 5.94-5.82 (1H, m, CH=CH₂ allyl), 5.50 (1H, d, $J = 7.9$ Hz, CH), 5.34 (1H, d, $J = 17.1$ Hz, CH=CH_{2trans}), 5.28 (1H, d, $J = 10.3$ Hz, CH=CH_{2cis}), 4.68-4.62 (2H, m, OCH₂ allyl), 3.84-3.77 (2H, m, OCH₂), 3.62 (2H, s, CH₂), 2.93-2.71 (2H, m, SCH₂); ¹³C NMR (75.4 MHz, CDCl₃): δ 170.8, 169.2, 134.9, 134.0, 131.0, 129.8, 129.6, 129.5, 129.2, 127.8, 127.7, 119.5, 66.8, 62.4, 53.2, 43.6, 34.6, 26.7; MS (ESI, positive mode): m/z 310.04 [M + H]⁺, calcd mass for C₁₅H₁₉NO₄S 309.10.

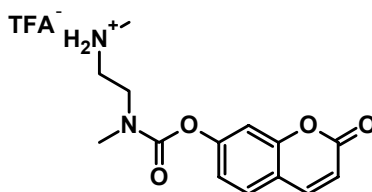
Model pro-fluorescent compound (VIII)



(a) Conversion into *N*-hydroxysuccinimide carbonate: Alcohol **VII** (18 mg, 58 μ mol) was dissolved in dry CH₃CN (2 mL). Then, *N,N'*-disuccinimidyl carbonate (DSC, 30 mg, 116

μmol) and DIEA (48 μL , 290 μmol) were sequentially added. The resulting reaction mixture was stirred at room temperature under a N_2 atmosphere for 8-9 h.

(b) Removal of the Boc group - synthesis of *N*-methylamine **IV** (TFA salt):

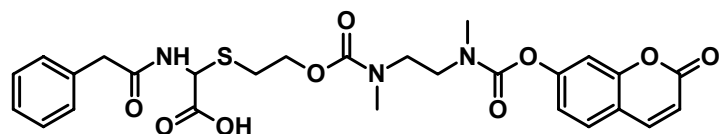


Coumarin **III** (44 mg, 0.116 mmol) was dissolved in TFA (0.6 mL) and the resulting solution was stirred at room temperature for 2 h. The reaction was checked for completion by TLC and TFA was removed under high vacuum to afford the TFA salt of *N*-methylamino coumarin **IV** in quantitative yield. The crude was dissolved in dry CH_3CN (1 mL) and used in the next step without further purifications.

(c) Coupling reaction:

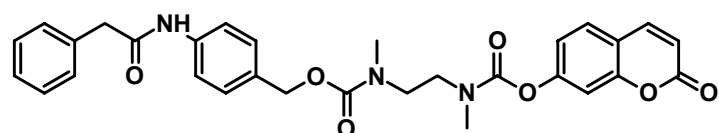
Solution of coumarin **IV** in CH_3CN and DIEA (20 μL) were sequentially added dropwise to the crude active carbonate mixture (see step a). The resulting mixture was stirred at room temperature overnight. The reaction was checked for completion by RP-HPLC (system A). After 15 h, the solvent was removed under vacuum and the residue was diluted in EtOAc. The organic phase was washed successively with aq. 10% NH_4Cl solution and deionised water. The organic layer was dried over Na_2SO_4 , filtered and evaporated. The residue was dissolved in a mixture of CH_3CN (1 mL) and deionised water (0.7 mL) and purified by semi-preparative RP-HPLC (System B). The product-containing fractions were lyophilised to give the PGA pro-fluorophore **VIII** as an hygroscopic white amorphous powder (11 mg, yield 32%). ^1H NMR (300 MHz, CDCl_3): δ 7.69-7.63 (1H, m, H_{coum}), 7.47-7.42 (1H, m, H_{coum}), 7.32-7.26 (5H, m, H_{Ar}), 7.11-6.87 (3H, m, H_{coum}), 6.37 (1H, m, NH), 5.93-5.80 (1H, m, $\text{CH}=\text{CH}_2$ allyl), 5.54 (1H, m, CH), 5.32 (1H, d, $J = 17.1$ Hz, $\text{CH}=\text{CH}_2$ trans), 5.24 (1H, d, $J = 10.3$ Hz, $\text{CH}=\text{CH}_2$ cis), 4.68-4.55 (2H, m, OCH_2 allyl), 4.24-4.19 (2H, m, OCH_2), 3.63-3.53 (6H, m, $\text{CH}_2 + \text{NCH}_2 + \text{CH}_2\text{N}$), 3.30-2.88 (8H, m, $\text{SCH}_2 + 2 \times \text{CH}_3$); ^{13}C NMR (75.4 MHz, CDCl_3): δ 170.8, 168.4, 160.8, 160.6, 156.0, 154.8, 154.2, 154.1, 154.0, 153.9, 143.2, 143.0, 134.3, 134.2, 131.1, 129.4, 128.7, 128.6, 128.5, 127.6, 119.4, 118.8, 118.6, 116.3, 115.8, 110.7, 110.5, 78.1, 66.8, 64.2, 63.9, 53.7, 53.6, 53.5, 47.6, 47.4, 47.2, 47.0, 46.9, 46.5, 46.3, 43.4, 43.3, 35.6, 35.5, 35.4, 35.3, 34.9, 34.7, 30.5, 30.3, 30.0; HPLC (system A): $t_{\text{R}} = 22.8$ min (purity 80%); UV (recorded during the HPLC analysis): λ_{max} 282, 309 nm; MS (ESI, positive mode): m/z 612.13 $[\text{M} + \text{H}]^+$, 1222.67 $[2\text{M} + \text{H}]^+$, calcd mass for $\text{C}_{30}\text{H}_{33}\text{N}_3\text{O}_9\text{S}$ 611.19.

Model pro-fluorescent compound (3)



Compound **VIII** (10 mg, 16 μmol) and $\text{Pd}(\text{PPh}_3)_4$ (1 mg, 10%) were dissolved in CHCl_3 under a N_2 atmosphere at room temperature. After 10 min, a solution of benzylamine (4 μL , 32 μmol) and dimedone (7 mg, 49 μmol) in dry CH_2Cl_2 was added at room temperature. The resulting reaction mixture was stirred at room temperature for 3 h (the color changed from colorless to yellow). The reaction was checked for completion by RP-HPLC (system A) and the mixture was filtered through a Celite[®] 545 pad and washed with CH_2Cl_2 . The solvent was evaporated and the residue was purified by semi-preparative RP-HPLC (system B). The product-containing fractions were lyophilised to give the PGA pro-fluorophore **3** as an hygroscopic white amorphous powder (8 mg, yield 89%). ^1H NMR (300 MHz, CDCl_3): δ 7.73-7.66 (1H, m, H_{coum}), 7.50-7.45 (1H, m, H_{coum}), 7.38-7.20 (5H, m, H_{Ar}), 7.10-6.92 (3H, m, H_{coum}), 6.42-6.36 (1H, m, NH), 5.64-5.49 (3H, m, CH + CH_2), 4.56-4.17 (2H, m, OCH_2), 3.62-3.53 (6H, m, CH_2 + NCH_2 + CH_2N), 3.13-2.71 (8H, m, SCH_2 + 2 x CH_3); ^{13}C NMR (75.4 MHz, CDCl_3): δ 171.3, 171.2, 169.3, 161.7, 161.6, 157.64, 157.1, 154.5, 154.1, 154.0, 143.7, 143.6, 143.3, 133.9, 129.4, 129.0, 128.8, 128.7, 127.6, 119.1, 118.8, 118.6, 116.5, 116.4, 115.8, 115.6, 110.6, 110.5, 63.6, 63.4, 63.1, 53.3, 47.3, 47.0, 46.9, 46.8, 46.4, 43.3, 43.2, 35.7, 35.4, 35.3, 35.0, 34.8, 34.6, 31.1, 30.8; HPLC (system A): t_{R} = 17.7 min (purity 96%); UV (recorded during the HPLC analysis): λ_{max} 282, 310 nm; MS (ESI, positive mode): m/z 572.07 [$\text{M} + \text{H}$]⁺; MS (ESI, negative mode): m/z 570.13 [$\text{M} - \text{H}$]⁻, 684.07 [$\text{M} + \text{TFA} - \text{H}$]⁻, calcd mass for $\text{C}_{27}\text{H}_{29}\text{N}_3\text{O}_9\text{S}$ 571.16.

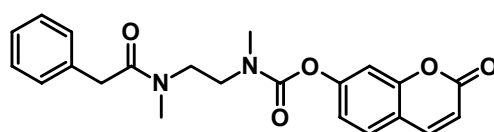
Model pro-fluorescent compound (5)



This compound was synthesised from *N*-[4-(hydroxymethyl)phenyl]-2-phenylacetamide⁴ and *N*-methylamino coumarin **IV** (TFA salt) using the procedure described for the preparation of PGA pro-fluorophore **VIII**. An hygroscopic white amorphous powder was obtained (8 mg,

yield 19%). ^1H NMR (300 MHz, CDCl_3): δ 7.69-7.64 (1H, m, H_{coum}), 7.46-7.26 (10H, m, $\text{H}_{\text{coum}} + 9\text{H}_{\text{Ar}}$), 7.01-6.89 (2H, m, H_{coum}), 6.69 (1H, s, NH), 6.39-6.33 (1H, m, H_{coum}), 5.10-5.03 (2H, m, CH_2O), 3.75 (2H, s, CH_2), 3.65-3.45 (4H, m, 2 x CH_2), 3.12-2.94 (6H, m, 2 x CH_3); HPLC (system A): $t_{\text{R}} = 21.2$ min (purity 96%); UV (recorded during the HPLC analysis): λ_{max} 250, 310 nm; MS (ESI, positive mode): m/z 544.07 $[\text{M} + \text{H}]^+$, 560.93 $[\text{M} + \text{H}_2\text{O}]^{++}$ (water cluster formed during the mass analysis), 566.13 $[\text{M} + \text{Na}]^+$, calcd mass for $\text{C}_{30}\text{H}_{29}\text{N}_3\text{O}_7$ 543.20.

Model pro-fluorescent compound (6)



Coumarin **III** was treated with TFA (800 μL) at room temperature for 30 min then evaporated to dryness. The resulting TFA salt was dissolved in dry CH_2Cl_2 and phenylacetyl chloride (71 μL , 0.53 mmol) and DIEA were sequentially added at 0 $^\circ\text{C}$. The resulting reaction mixture was allowed to warm to room temperature and stirred for 30 min. Upon completion, the reaction was diluted with CH_2Cl_2 and washed successively with saturated aq. NH_4Cl solution and deionised water. The organic layer was dried over Na_2SO_4 and the solvent was removed under reduced pressure. The resulting residue was purified by chromatography on a silica gel column using a mixture of (cyclohexane-EtOAc, 2 : 8, v/v) as the mobile phase to give PGA pro-fluorophore **6** as a colorless oil (90 mg, yield 88%). R_{f} 0.12 (cyclohexane/EtOAc, 2 : 8, v/v); ^1H NMR (300 MHz, $\text{CDCl}_3 + \text{CH}_3\text{OH}$ for calibration): δ 7.70 (1H, d, $J = 9.6$ Hz, H_{coum}), 7.50-7.40 (1H, m, H_{coum}), 7.35-7.23 (5H, m, H_{Ar}), 7.14-6.99 (2H, m, H_{coum}), 6.39 (1H, d, $J = 9.6$ Hz, H_{coum}), 3.80-3.52 (6H, m), 3.48-3.00 (6H, m); ^{13}C NMR (75.4 MHz, CDCl_3): δ 171.7, 171.5, 171.3, 171.0, 160.5, 154.6, 154.1, 154.0, 153.9, 153.4, 143.0, 142.9, 135.0, 134.7, 134.4, 134.3, 129.3, 128.9, 128.8, 128.7, 128.5, 128.4, 126.9, 126.8, 118.6, 118.4, 116.2, 115.8, 115.6, 110.4, 110.3, 110.1, 48.0, 47.5, 47.4, 46.6, 46.1, 45.1, 41.2, 40.9, 40.7, 40.6, 36.5, 36.1, 35.8, 35.5, 35.1, 34.2, 33.9, 29.7; HPLC (system A): $t_{\text{R}} = 16.8$ min (purity >92%); UV (recorded during the HPLC analysis): λ_{max} 282, 310 nm; MS (ESI, positive mode): m/z 395.20 $[\text{M} + \text{H}]^+$, 411.93 $[\text{M} + \text{H}_2\text{O}]^{++}$ (water cluster formed during the mass analysis), 417.20 $[\text{M} + \text{Na}]^+$, calcd mass for $\text{C}_{22}\text{H}_{22}\text{N}_2\text{O}_5$ 394.15.

General procedure for *in vitro* cleavage of PGA pro-fluorophore 3-6 by penicillin G acylase (PGA)

Fluorescence assay:

Mixture a: A stock solution (7.28 mM) of PGA pro-fluorophore in DMSO was prepared.

Mixture b: 2.22 mg of PGA (0.63 U/mg) was dissolved in 1 mL of PBS (100 mM phosphate, 150 mM NaCl, pH 7.5).

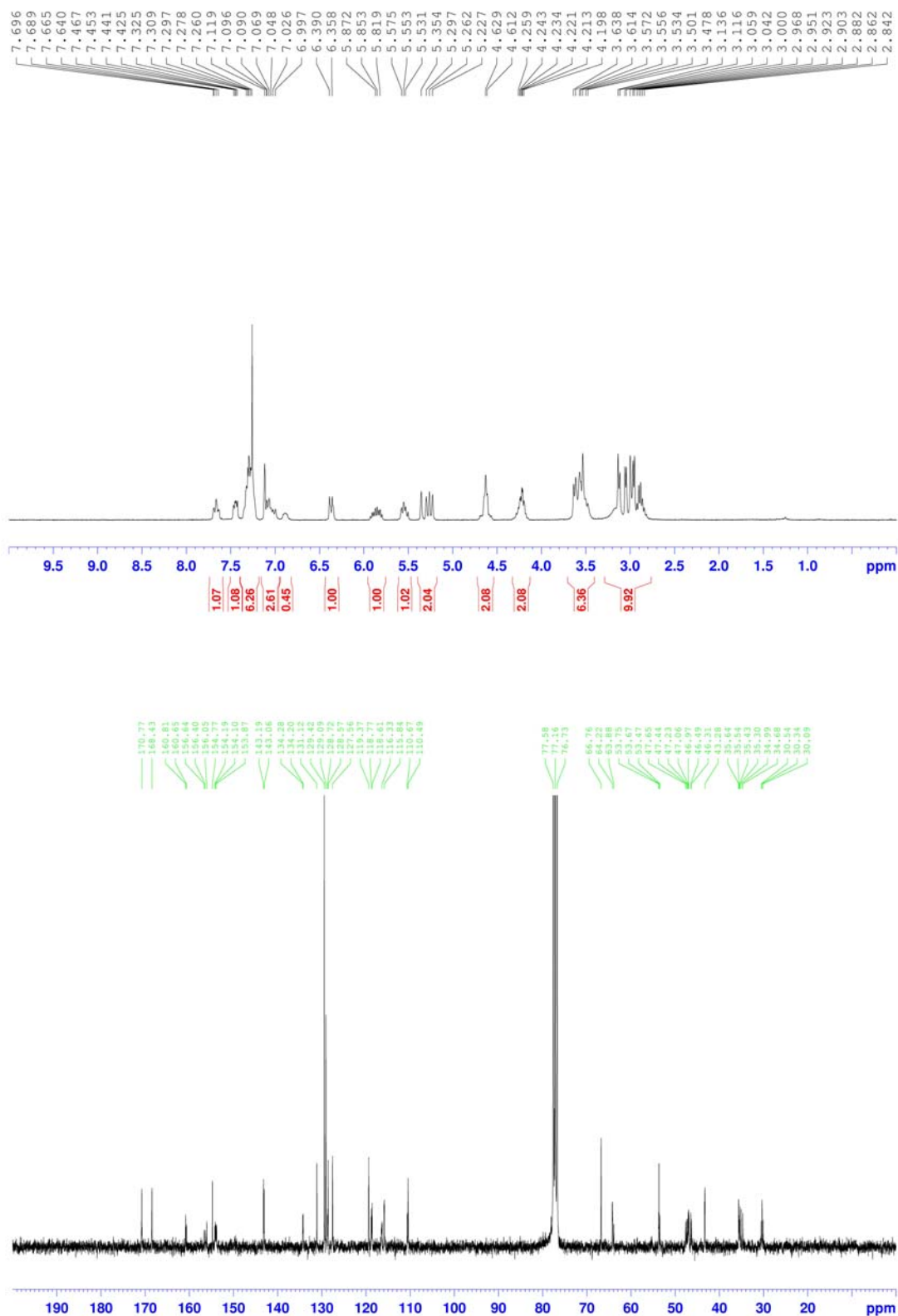
To the 1.5 mL fluorescence cuvette (Hellma, 104F-QS, 10 × 4 mm, 1400 μL), 1313 μL of PBS, 0.57 μL of **mixture a**, and 85.7 μL of **mixture b** were sequentially added. The resulting enzymatic reaction mixture was homogenised by magnetic stirring for 30 s, thermostated at 37 °C and the fluorescence emission of the released 7-hydroxycoumarin was monitored at $\lambda = 460$ nm (emission slit = 5 nm) (Ex. $\lambda = 360$ nm, excitation slit = 5 nm) over time with measurements recorded every 20 s.

The non-specific hydrolysis was monitored by fluorescence emission measurements at $\lambda = 460$ nm without PGA enzyme in the same conditions.

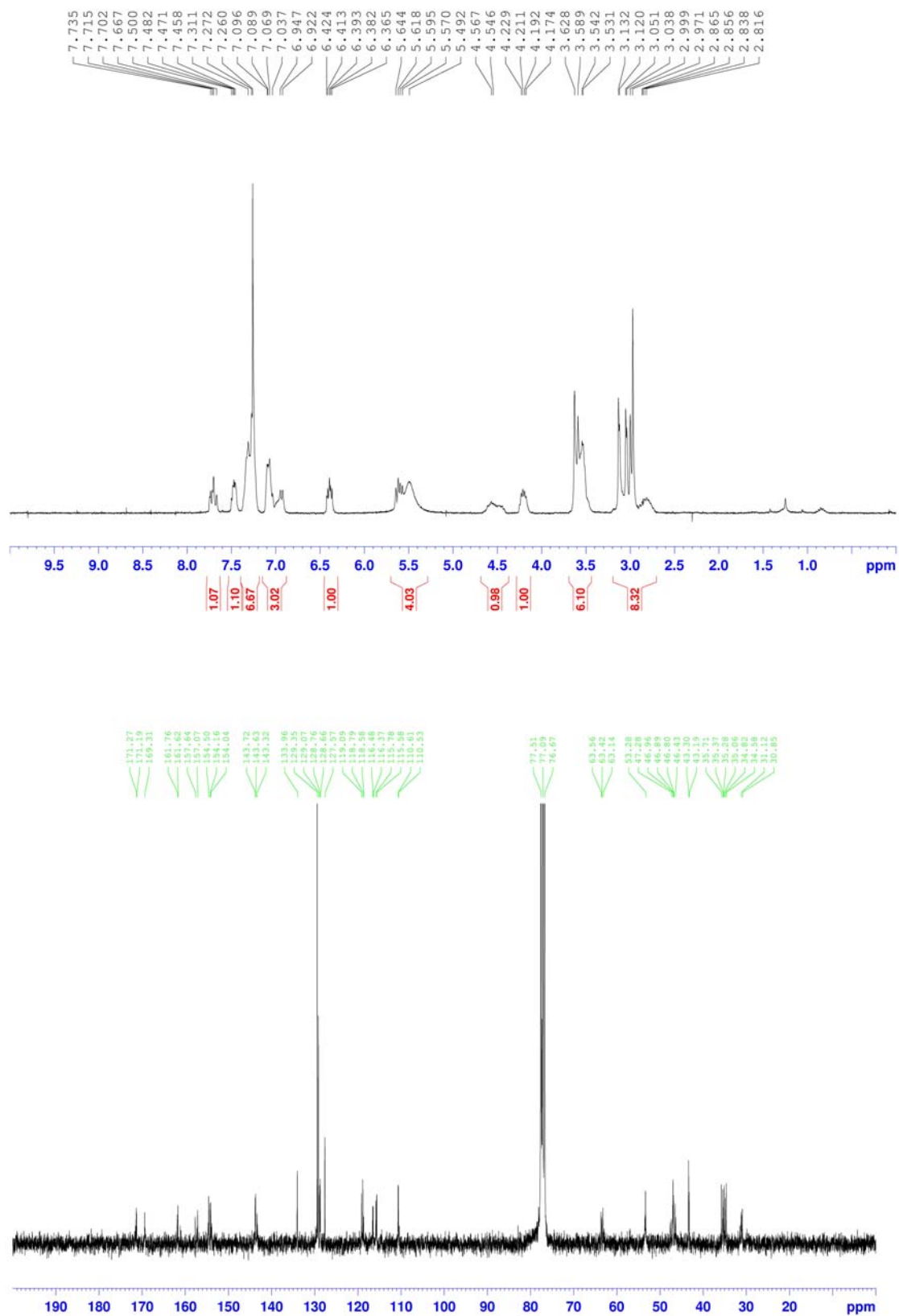
References

- 1 H. E. Gottlieb, V. Kotlyar and A. Nudelman, *J. Org. Chem.*, 1997, **62**, 7512.
- 2 M. Shamis and D. Shabat, *Chem. Eur. J.*, 2007, **13**, 4523.
- 3 Y. Meyer, J. A. Richard, M. Massonneau, P. Y. Renard and A. Romieu, *Org. Lett.*, 2008, **10**, 1517.
- 4 J. A. Richard, Y. Meyer, V. Jolivel, M. Massonneau, R. Dumeunier, D. Vaudry, H. Vaudry, P. Y. Renard and A. Romieu, *Bioconjugate Chem.*, 2008, **19**, 1707.

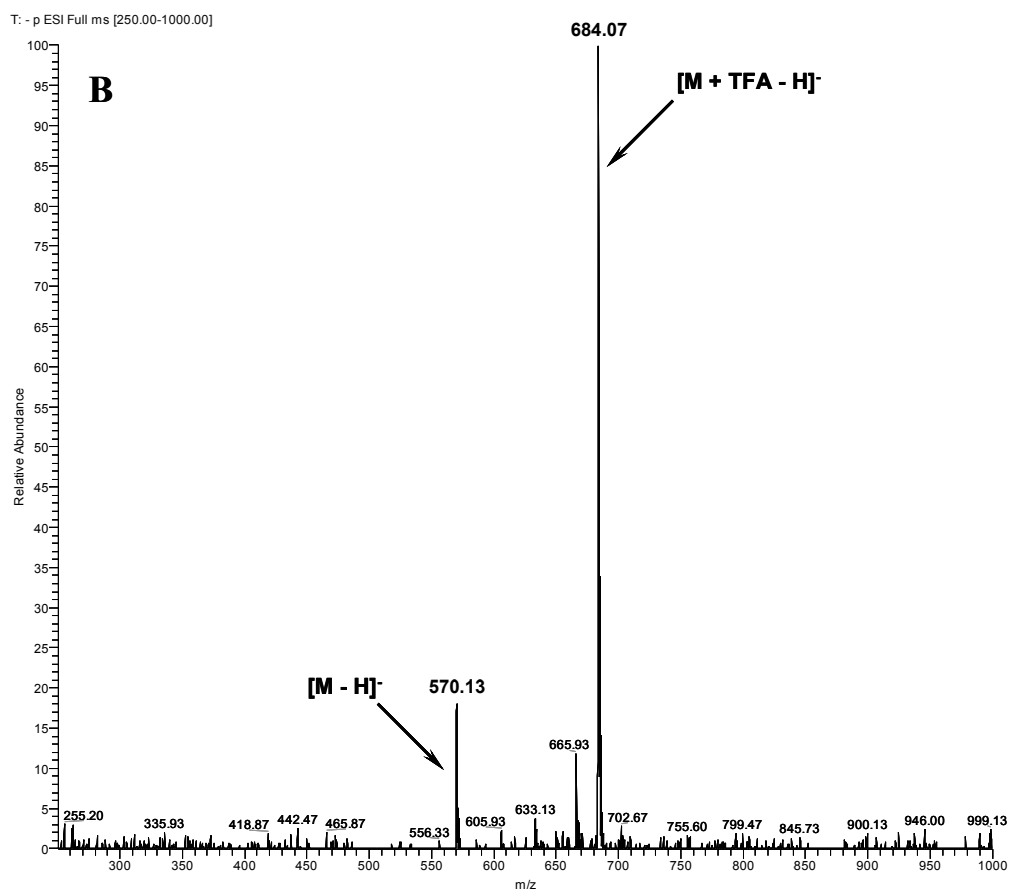
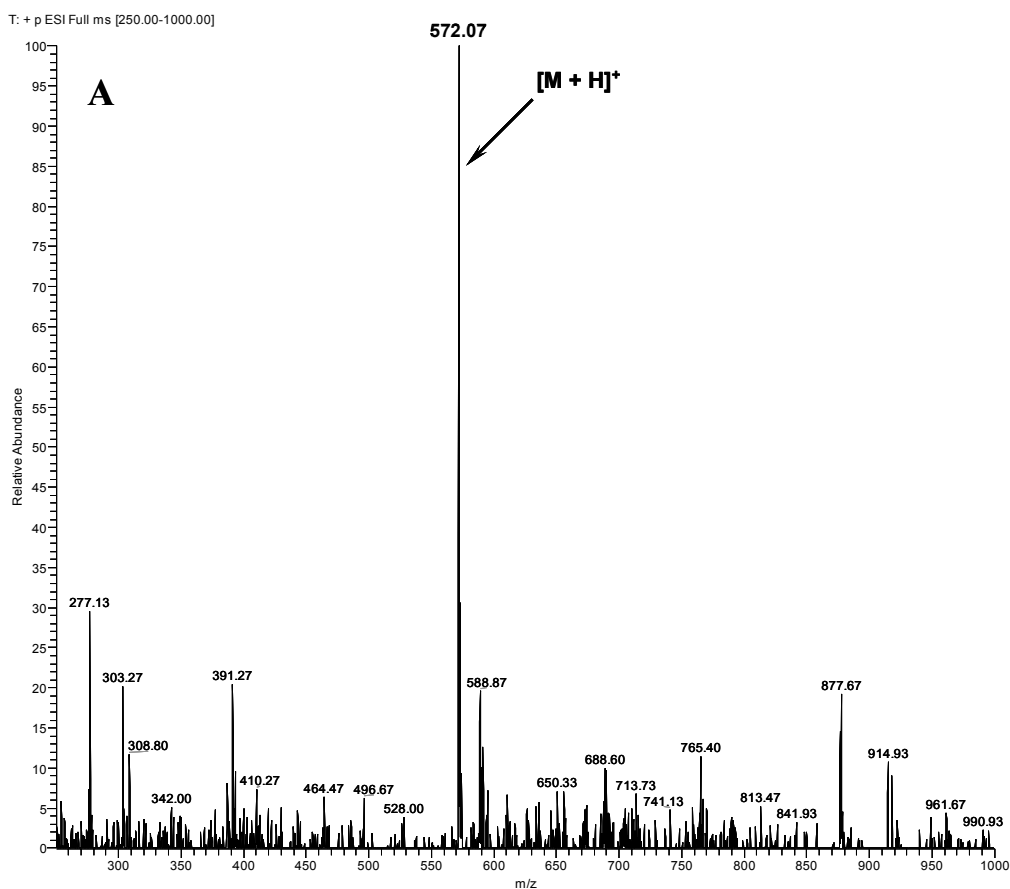
^1H and ^{13}C NMR spectra of PGA pro-fluorophore VIII recorded in CDCl_3



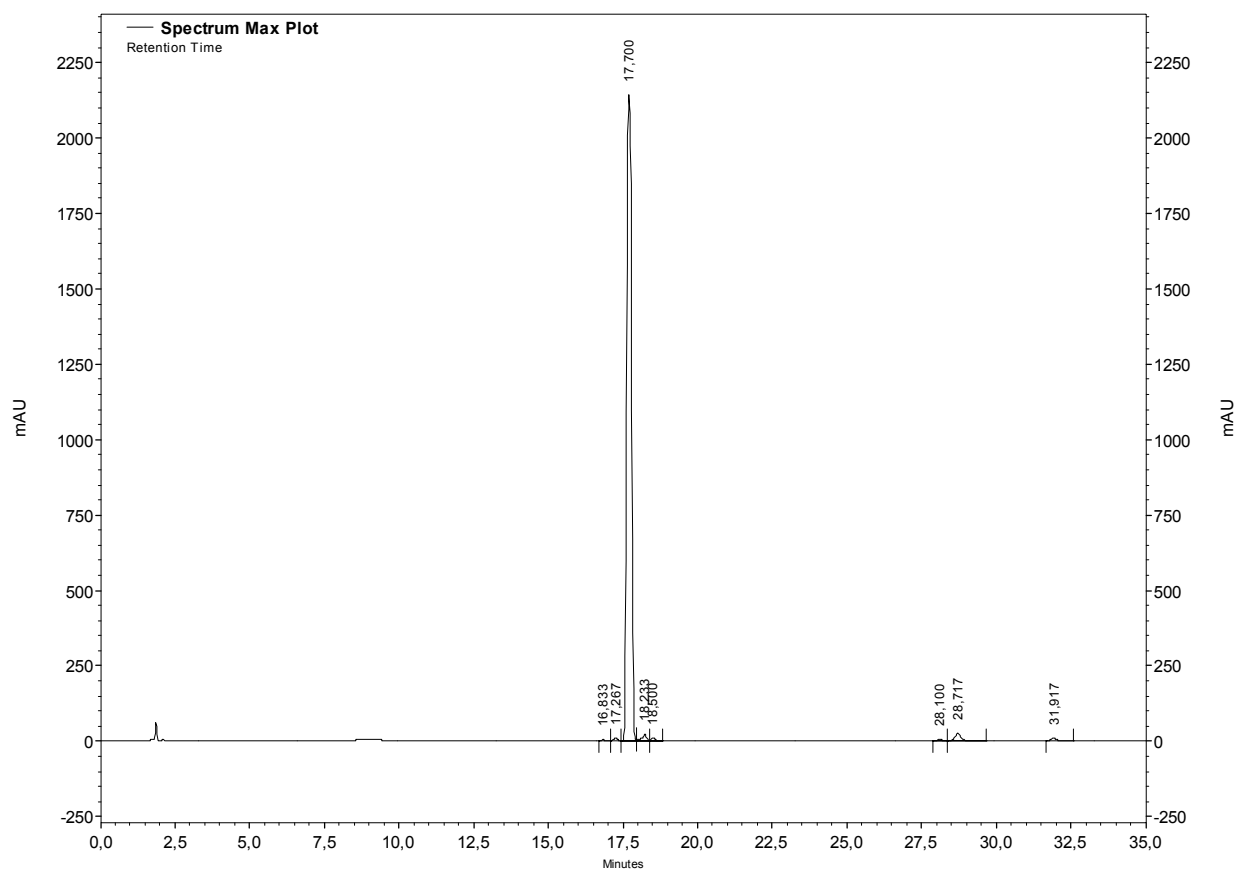
^1H and ^{13}C NMR spectra of PGA pro-fluorophore **3** recorded in CDCl_3



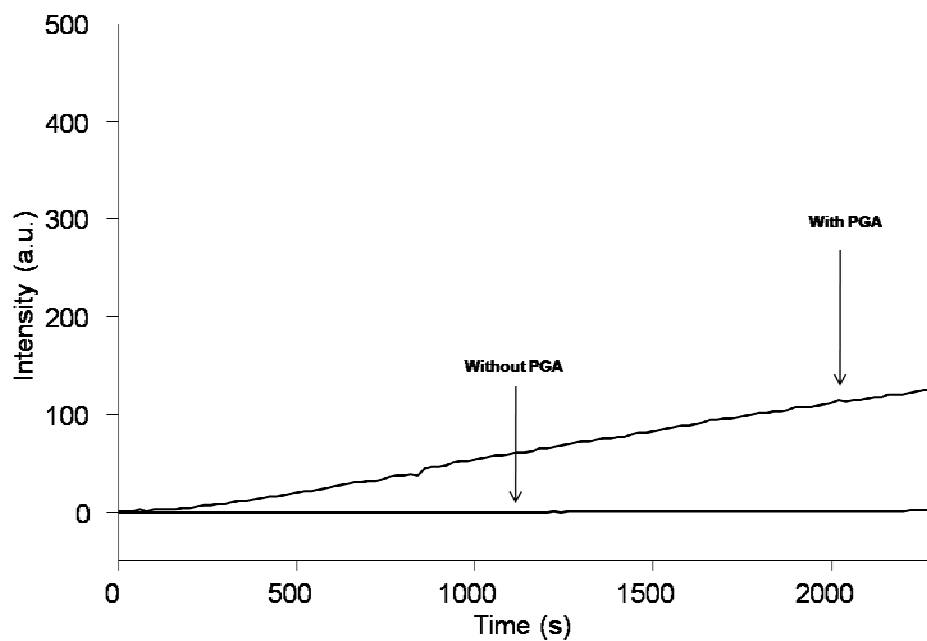
ESI-MS spectra of PGA pro-fluorophore 3 recorded in the positive (A) and negative (B) modes



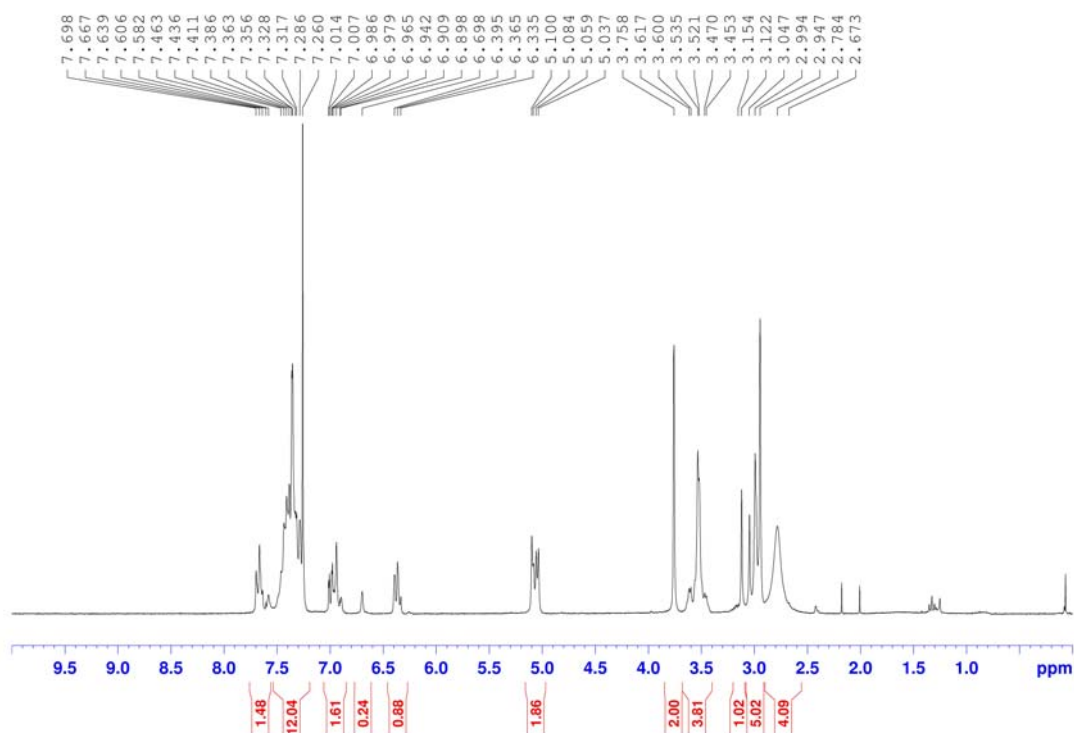
RP-HPLC elution profile of PGA pro-fluorophore 3 (system A)



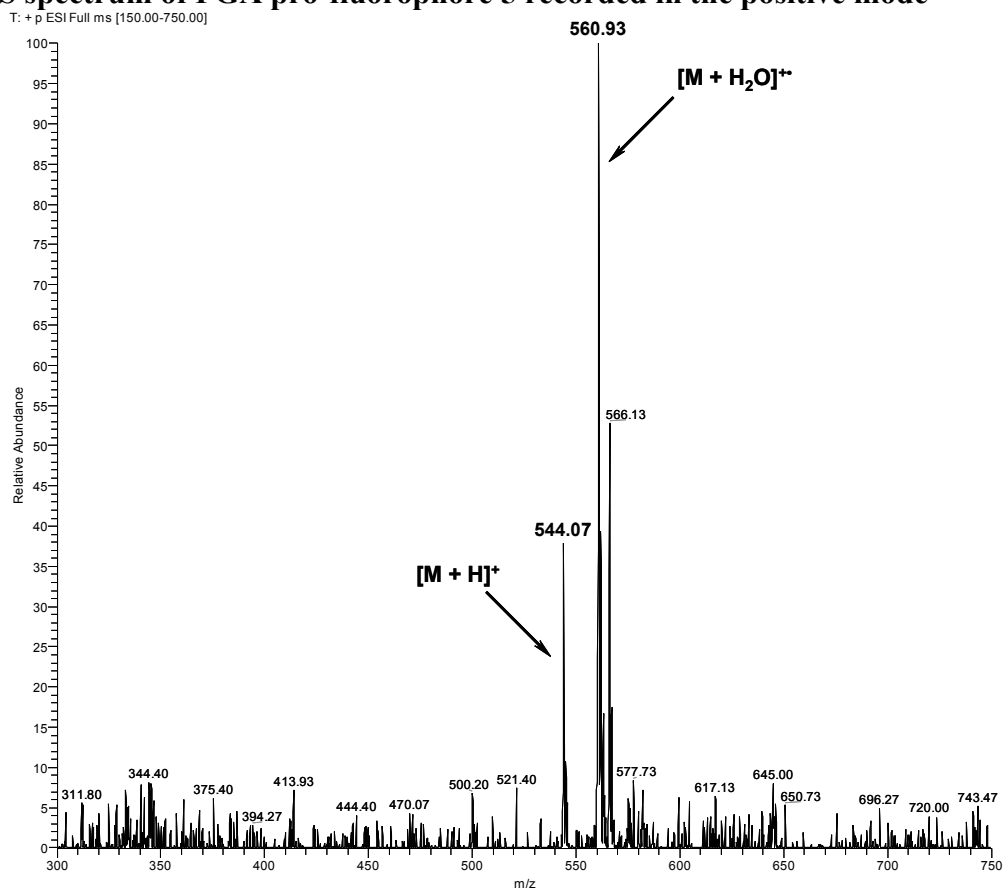
Fluorescence emission time-course of PGA pro-fluorophore 3



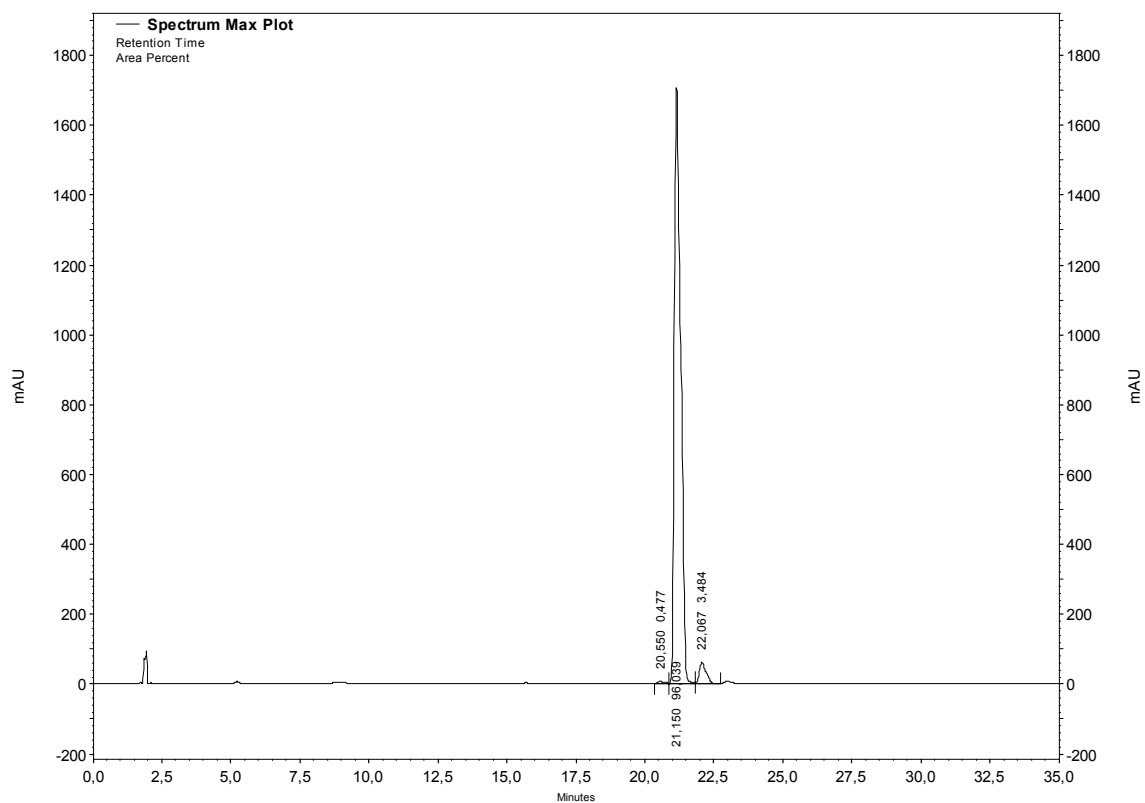
^1H NMR spectrum of PGA pro-fluorophore 5 recorded in CDCl_3



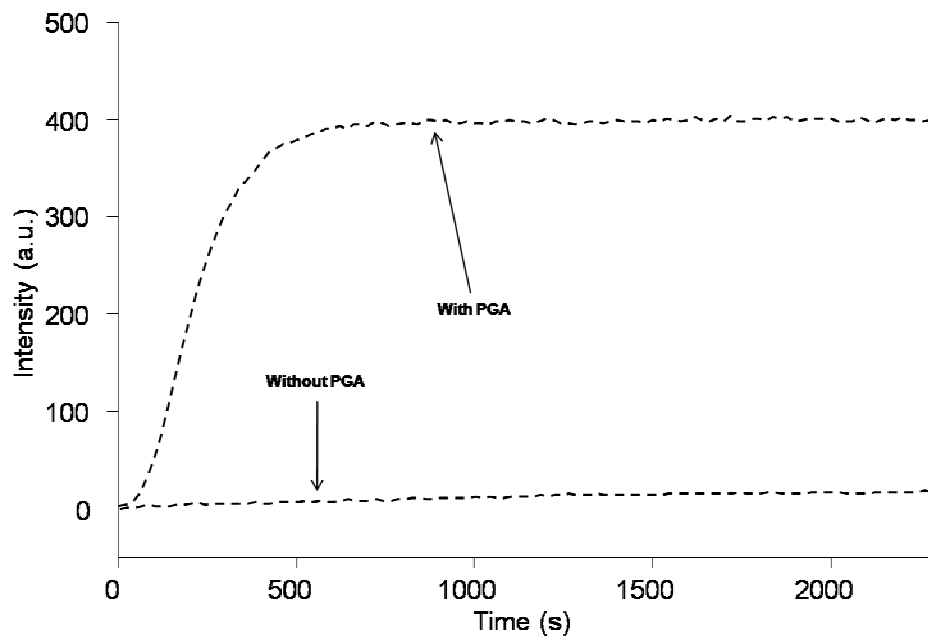
ESI-MS spectrum of PGA pro-fluorophore 5 recorded in the positive mode



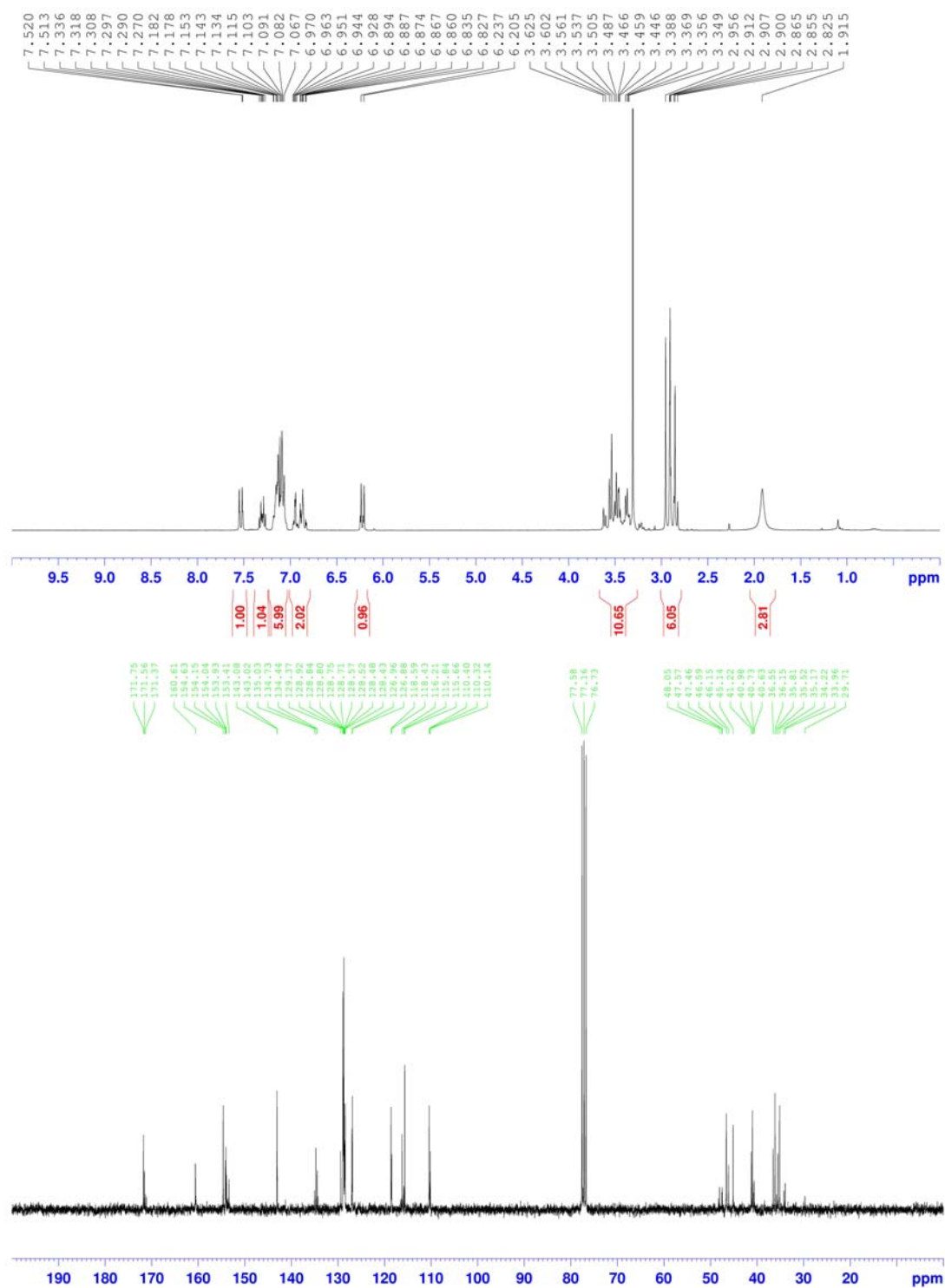
RP-HPLC elution profile of PGA pro-fluorophore 5 (system A)



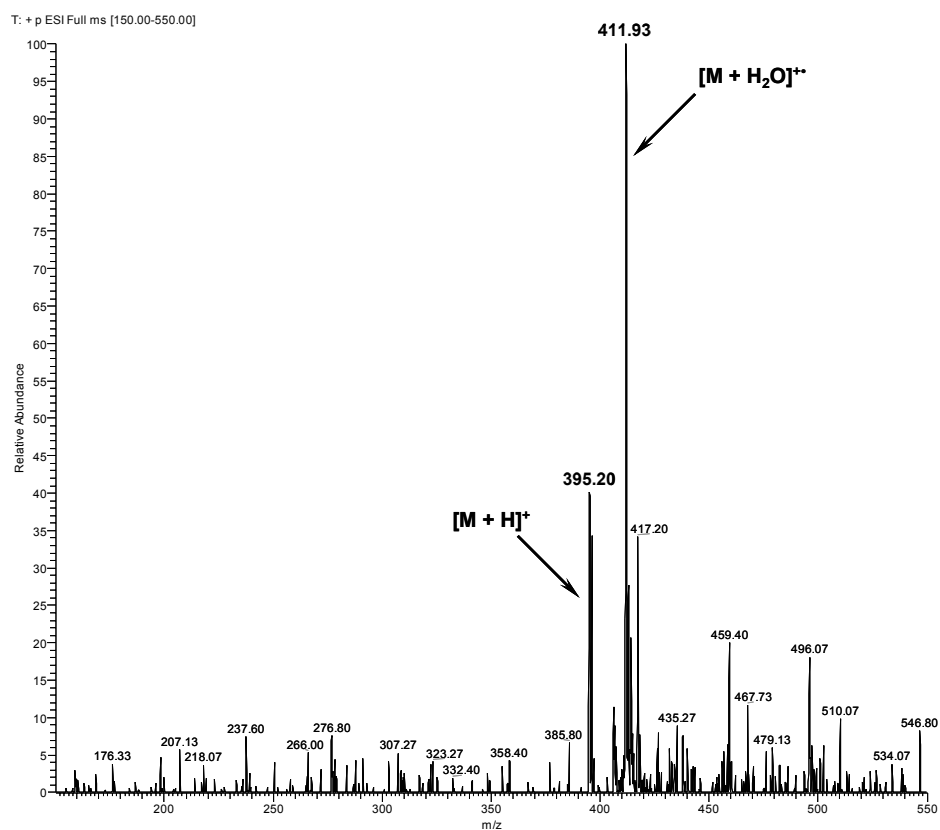
Fluorescence emission time-course of PGA pro-fluorophore 5



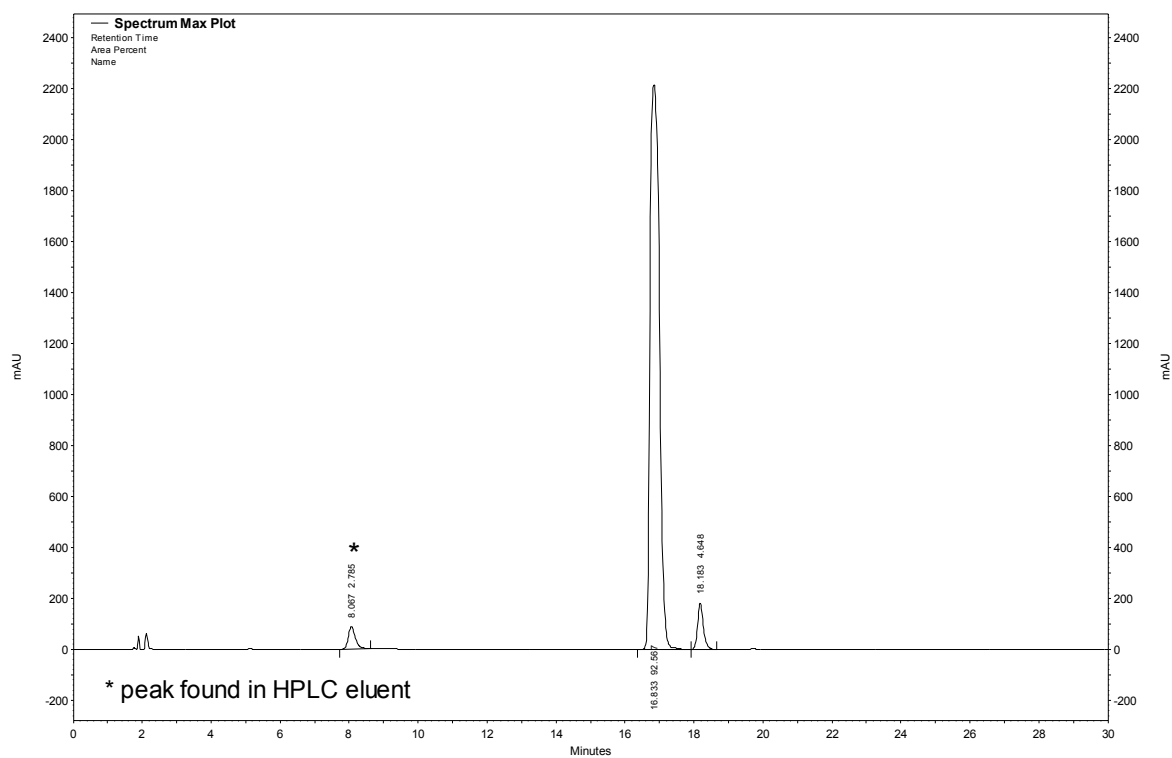
^1H and ^{13}C NMR spectra of PGA pro-fluorophore **6** recorded in CDCl_3



ESI-MS spectrum of PGA pro-fluorophore 6 recorded in the positive mode



RP-HPLC elution profile of PGA pro-fluorophore 6 (system A)



Fluorescence emission time-course of PGA pro-fluorophore 6

