Synthesis of New Hydrophilic Rhodamine Based Enzymatic Probes Compatible With Droplet-Based Microfluidic Assays.

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Supplementary Information

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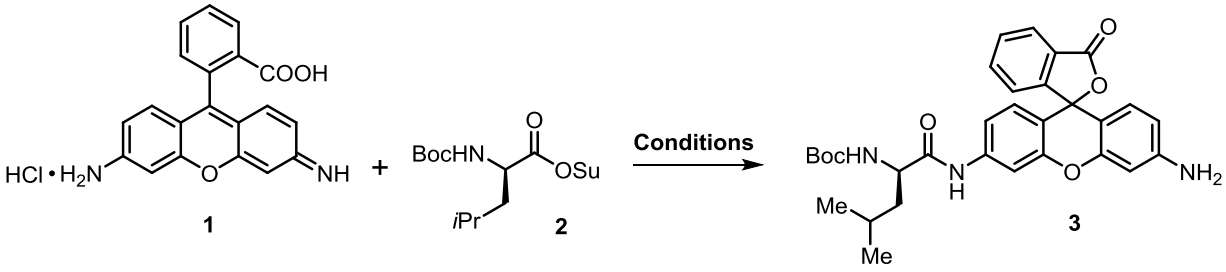
General information

All reactions were carried out under an argon atmosphere unless otherwise specified. Flasks were oven-dried at 120 °C and cooled under argon prior to use. CH_2Cl_2 was distilled over calcium hydride. All others commercially available chemicals were purchased from Aldrich and used directly without purification. TLCs were performed on Merck $60F_{254}$ silica gel plates and visualized with UV lamp (254 or 360 nm), and by treatment with a solution of $KMnO_4$, K_2CO_3, NaOH (5%) in H_2O followed by heating. Reverse phase TLC were performed on Merck 60 RP-18 F_{254} silica gel plates and visualized with UV lamp (254 or 360 nm). Flash column chromatographies were performed with Merck Geduran Si 60 silica gel (40-63 μm). Reverse phase chromatography were performed using a Biotage Isolera One flash purification system using Biotage SNAP cartridge KP-C18-HS 12g and eluted with HPLC grade MeCN/H_2O. The fractions were collected using UV detection at 254 and 360 nm and concentrated under reduced pressure at 40 °C, residual water was removed *via* lyophilisation.^{1}H NMR spectra were recorded on a Bruker Avance 400 at 400 MHz. The chemical shifts δ are reported in ppm relative to tetramethylsilane (TMS) or residual protonated solvents (TMS: δ_H = 0.00 ppm, $CHCl_3$: δ_H = 7.26 ppm, MeOD: δ_H = 3.31 ppm) The multiplicity and shape of signals are designated by the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet, m = multiplet, br = broad. Coupling constants *J* are reported in Hertz (Hz).^{13}C NMR spectra were recorded on a Bruker Avance 400 at 100 MHz. The chemical shifts δ are reported in ppm relative to the solvent as an internal indicator ($CDCl_3$ *δ* 77.16 ppm, CD_3OD *δ* 49.00 ppm). IR-spectra were recorded on a Bruker TENSOR^{TM} 27 (IRTF). The samples were prepared as neat films or as fine powders. Only selected absorbances (ν_{max}) are reported, and wave numbers are reported in cm^{-1}. High resolution mass spectra (HRMS) were performed by the Centre Regional de Microanalyse (Université Pierre et Marie Curie VI, Paris, France).

Optimization of conditions for compound 3:

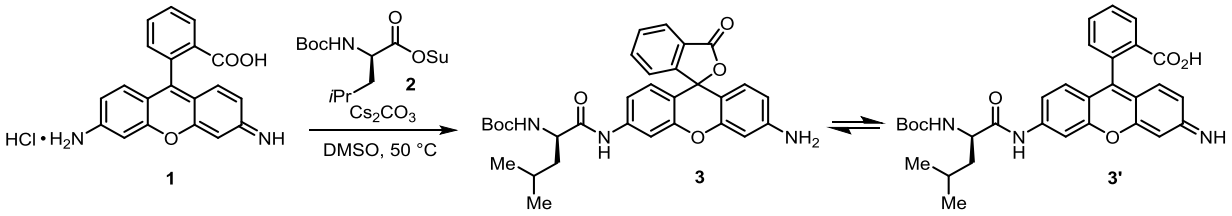


Reagent	Base	Solvent	Temperature	Yield [a]
2, (1.1 eq.)	K_2CO_3 (5 eq.)	DMF/H_2O	23 °C	12% [b]
2, (1.5 eq.)	Pyridine (excess)	DMF	23 °C	-
2, (1.5 eq.)	Cs_2CO_3 (1 eq.)	DMF/Pyridine	23 °C	18% [c]
2, (1.1 eq.)	Et_3N (3 eq.)	1,4-dioxane	50 °C	29%
2, (1.5 eq.)	Et_3N (2 eq.)	DMSO	23 °C	27%
2, (1.5 eq.)	Cs_2CO_3 (3 eq.)	DMSO/H_2O	23 °C	< 10% [d]
2, (1.5 eq.)	Cs_2CO_3 (3 eq.)	DMSO	23 °C	37%
2, (1.5 eq.)	Cs_2CO_3 (3 eq.)	DMSO	50 °C	53%
2, (1.5 eq.)	-	DMSO	23 °C	-

[a] Isolated yield after purification. [b] DMF/H_2O (9/1) mixture was used, [c] DMF/Pyridine (3/1) mixture was used, [d] DMSO/H_2O (9/1) mixture was used. All the reactions were performed on a 0.1 mmol scale at 0.1 M concentration.

Experimental procedure

***tert*-Butyl ((2*R*)-1-((3'-amino-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)amino)-4-methyl-1-oxopentan-2-yl)carbamate(3):**



To a solution of rhodamine 110 hydrochloride (40 mg, 0.109 mmol, 1 eq) in DMSO (1 mL) was added Cs_2CO_3 (106 mg, 0.327 mmol, 3 eq) followed by *N*-Boc-L-leucine *N*-hydroxysuccinimide ester (53 mg, 0163 mmol, 1.5 eq) and heated at 50 °C for 12 h. The mixture was then dilute with EtOAc (40 mL), washed with H_2O (3 x 10 mL), brine (3 x 10 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. The resulting orange oil was purified by flash chromatography on silica gel 10:90 to 30:70 (EtOAc/$CHCl_3$) to afford compound **3** as an amorphous orange solid (31 mg, 0.057 mmol, 53%).

This compound exists as an equilibrium mixture of **3** and its open form **3'** in $CDCl_3$.

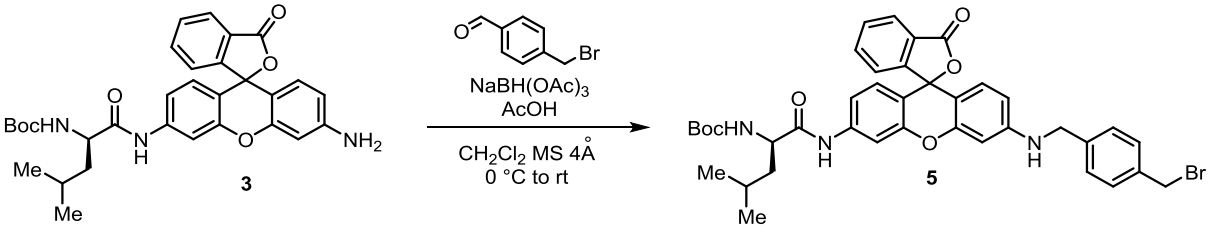
^{1}H NMR (400 MHz, $CDCl_3$) *Described as 1/0.9 mixture of tautomers: Major tautomer* δ 8.74 (brs, 1H), 8.00 (s, 1H), 7.69 (d, *J* = 2.1 Hz, 1H), 7.65 – 7.55 (m, 2H), 7.07 (d, *J* = 7.7 Hz, 1H), 6.99 (dd, *J* = 8.6, 2.2 Hz, 1H), 6.67 (d, *J* = 2.5 Hz, 1H), 6.51 (s, 1H), 6.49 (d, *J* = 2.3 Hz, 1H), 6.33 (t, *J* = 2.0 Hz, 1H), 5.05 (d, *J* = 8.1 Hz, 1H), 4.26 (brs, 1H), 3.91 (brs, 2H),1.80 – 1.70 (m, 2H), 1.62 – 1.52 (m, 1H), 1.44 (s, 9H), 0.96 (t, *J* = 6.7 Hz, 6H) ppm. *Minor tautomer* δ 8.74 (brs, 1H), 7.98 (brs, 1H), 7.66 (d, *J* = 2.1 Hz, 1H), 7.65 – 7.55 (m, 2H), 7.10 (d, *J* = 7.7 Hz, 1H), 6.95 (dd, *J* = 8.6, 2.2 Hz, 1H), 6.65 (d, *J* = 2.5 Hz, 1H), 6.53 (s, 1H), 6.45 (brs, 1H), 6.31 (t, *J* = 2.0 Hz, 1H), 5.05 (d, *J* = 8.1 Hz, 1H), 4.26 (brs, 1H), 3.91 (brs, 2H), 1.80 – 1.70 (m, 2H), 1.62 – 1.52 (m, 1H), 1.44 (s, 9H), 0.96 (t, *J* = 6.7 Hz, 6H) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) *Described as 1/0.9 mixture of tautomers* δ [171.89, 171.83], 169.97, 156.56, 153.29, [152.55, 152.51], 151.86, [149.11, 149.07], 139.92, [135.16, 135.12], [129.70, 129.67], [129.00, 128.96], 128.50, [126.87, 126.83], 124.94, [124.13, 124,13], [115.32, 115.13], [114.61, 114.46], [111.74, 11.69], [108.24, 108.21], [107.93, 107,81], [101.63, 101, 58], 83.95, 80.69, 54.11, 40.91, [28.47, 28.45], 24.88, [23.14, 23.12], 21.89 ppm.

IR (neat): 3306, 2957, 2926, 2854, 1744, 1672, 1613, 1534, 1508, 1366, 1286, 1249, 1208 1168, 1113, 870 cm^{-1}.

ESI-HRMS: *m*/*z* calculated for $C_{31}H_{34}N_3O_6$ $[M+H]^+$: 544.2442, found: 544.2436.

***tert*-Butyl ((2*R*)-1-((3'-((4-(bromomethyl)benzyl)amino)-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)amino)-4-methyl-1-oxopentan-2-yl)carbamate (5)**:



To a solution of **3** (170 mg, 0.324 mmol, 1 eq) and 4-bromomethyl benzaldehyde[1] (77 mg, 0.389 mmol, 1.2 eq) in CH_2Cl_2 (5 mL) was added activated 4 Å MS (200 mg), $NaBH(OAc)_3$ (109 mg, 0.518 mmol, 1.6 eq) followed by the addition of AcOH (100 µL). The solution turned from orange to red and the flask was covered from the light with aluminium foil. After 3 h, an aqueous K_2CO_3 solution (10%) was added and the reaction mixture was extracted with CH_2Cl_2 (3 x 10 mL). The combined organic phases were dried over $MgSO_4$, filtered, concentrated under reduced pressure and purified by flash chromatography (protected from light with aluminium foil) on silica gel 10:90 to 20:80 (EtOAc/$CHCl_3$) to afford the unstable and light sensitive compound **5** (189 mg, 0.260 mmol, 79%) as a red oil.

This compound is unstable and light sensitive and has to be kept in the dark and engaged in the next step within 2 h, otherwise extensive decomposition was observed.

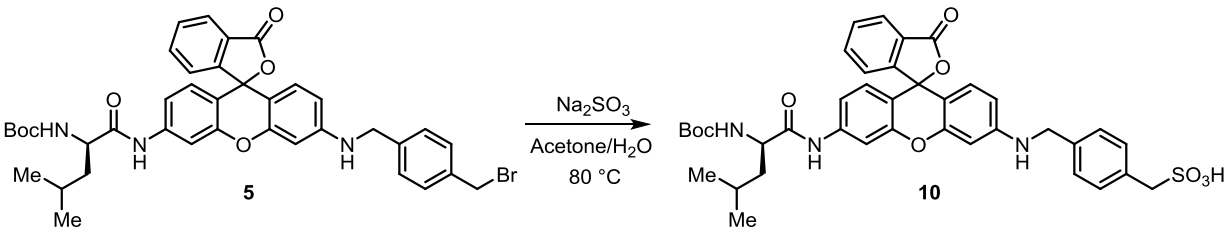
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.94 (brd, 1H), 7.98 (m, 1H), 7.71 – 7.52 (m, 3H), 7.36 – 7.26 (m, 4H), 7.02 (m, 2H), 6.62 (d, J = 8.6 Hz, 1H), 6.47 (d, J = 8.6 Hz, 1H), 6.37 – 6.23 (m, 2H), 5.18 (brs, 1H), 4.47 (s, 2H), 4.36 – 4.23 (m, 3H), 4.28 (brs, 1H), 1.77 – 1.66 (m, 2H), 1.62 – 1.53 (m, 1H), 1.43 (s, 9H), 0.98 – 0.81 (m, 6H) ppm.

IR (neat): 3307, 2958, 2929, 2854, 1747, 1674, 1613, 1510, 1413,1366, 1335,1285, 1250, 1166, 1124, 1224, 1084, 1020, 870 cm^{-1}.

ESI-HRMS: *m*/*z* calculated for $C_{39}H_{41}BrN_3O_6$ $[M+H]^+$: 726.2173, found: 726.2173.

[1]Prepared according procedure described by : L. Wen, M. Li and J. B. Schlenoff, *J. Am. Chem. Soc*. 1997, **119**, 7726.

Sodium (4-(((3'-((*R*)-2-((*tert*-butoxycarbonyl)amino)-4-methylpentanamido)-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)amino)methyl)phenyl)methanesulfonate (10):



To a solution of bromide **5** (152 mg, 0.209 mmol, 1 eq) in acetone (4 mL) was added a solution of Na_2SO_3 (131 mg, 1.04 mmol, 5 eq) in H_2O (2 mL). The mixture was then heated at 80 °C for 1 h under vigorous stirring. The mixture was then cooled to room temperature, concentrated and engaged in the next step without further purification.

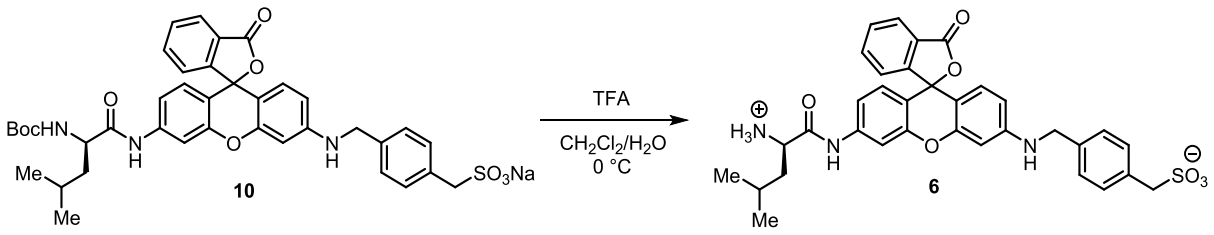
An aliquot (15 mg) was purified with reverse phase silica gel chromatography (40:60 to 60:40 MeCN/H_2O + 0.1% TFA) to afford, after lyophilisation, pure sulfonate **10** (4 mg) for characterisation purpose.

^{1}H NMR (400 MHz, MeOD) δ 8.32 (m, 2H), 7.85 (m, 2H), 7.48 – 7.37 (m, 4H), 7.37 – 7.30 (m, 2H), 7.20 (m, 1H), 7.11 (m, 1H), 7.03 – 6.86 (m, 2H), 4.63 (s, 2H), 4.28 (m, 1H), 4.04 (s, 2H), 1.75 (m, 1H), 1.68 – 1.50 (m, 2H), 1.45 (s, 9H), 0.99 (s, 3H), 0.97 (s, 3H) ppm. *-SO_3H and -NH protons(x3) were not observed in these conditions*.

IR (neat): 3326, 2963, 2926, 2854, 2362, 1744, 1684, 1614, 1517, 1414, 1366, 1286, 1249, 1168, 1120, 873 cm^{-1}.

ESI-HRMS: *m*/*z* calculated for $C_{39}H_{42}N_3O_9S$ $[M+H]^+$: 728.2636, found: 728.2639.

(4-(((3'-((*R*)-2-ammonio-4-methylpentanamido)-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)amino)methyl)phenyl)methanesulfonate (6):



The crude sulfonate **10** was dissolved in CH_2Cl_2/H_2O (10:1 - 2.2 mL) at 0 °C and TFA (2 mL) was added dropwise to the mixture. After 0.5 h the mixture was carefully concentrated under reduced pressure. The residue was then adsorbed on C18 SiO_2, using MeCN, and twice purified on reverse phase silica gel chromatography (20:80 to 50:50 $MeCN/H_2O$ + 0.1% TFA) to obtain pure sulfonate **6** (49 mg, 78.1 μmol, 37% over two steps after lyophilisation).

The purity of **6** was measured to be >95% by UV (at 254 nm) and ^{1}H NMR.

^{1}H NMR (400 MHz, MeOD) δ 8.33 (m, 2H), 7.91 – 7.74 (m, 2H), 7.52 – 7.46 (m, 1H), 7.44 (d, *J* = 8.1 Hz, 1H), 7.39 (d, *J* = 7.3 Hz, 2H), 7.34 (d, *J* = 8.1 Hz, 2H), 7.22 (d, *J* = 9.1 Hz, 1H), 7.12 (d, *J* = 9.3 Hz, 1H), 6.98 (d, *J* = 9.3 Hz, 1H), 6.90 (s, 1H), 4.64 (s, 2H), 4.12 (t, *J* = 6.8 Hz, 1H), 4.05 (s, 2H), 1.87 – 1.72 (m, 3H), 1.05 (d, *J* = 5.5 Hz, 3H), 1.04 (d, *J* = 5.5 Hz, 3H) ppm. *–NH protons (x 5) were not observed in these conditions*.

^{13}C NMR (100 MHz, MeOD) δ 170.5, 168.3, 161.0, 160.8, 156.0, 146.6, 136.7, 134.4 (2C), 132.8, 132.2, 132.0, 131.8, 131.7, 131.1, 130.7, 128.5, 120.7, 119.3, 119.1, 117.9, 117.7, 107.8, 96.8, 58.1, 54.0, 48.1, 41.4, 38.2, 23.3, 21.8 ppm. *One C^{IV} is not observed due to relaxation.*

IR (neat): 2965, 2854, 2360, 1680, 1599, 1557, 1512, 1445, 1340, 1259, 1051, 1014, 1202, 1136, 1033 cm^{-1}.

ESI-HRMS: *m/z* calculated for $C_{34}H_{34}N_3O_7S$ $[M+H]^+$: 628.2112, found: 628.2107

Microfabrication:

All microfluidic chips were fabricated in PDMS using soft-lithography techniques (J. Cooper McDonald *et al.*[3]) according to the protocol already described by Mazutis *et al.*[2]. If not stated otherwise, microfluidic channels are 20 µm deep.

Enzymatic assays and substrate exchange experiments:

Purified Aminopeptidase I from SGAP was purchased from Sigma Aldrich (A9934 SIGMA) and dissolved in SGAP buffer (2 mM $CaCl_2$, 200 µM $ZnCl_2$, 40 mM Tricine, pH = 8.0) to a concentration of 1 mg/mL. Solid substrate was dissolved in SGAP buffer to a concentration of 10 µM. For each substrate (**6** or **8**), a positive assay and a negative assay (no enzyme) were performed at 37 °C. In each well, 1 µL of SGAP solution or 1 µL of enzymatic buffer was added to 99 µL of substrate in enzymatic buffer (10 µM final substrate concentration). Evolution of the fluorescence was recorded in a fluorescence plate reader (SpectraMax i3x, Molecular Devices) with the following spectral settings: λ_{ex}= 488 nm, λ_{em}= 535 nm.

[2] L. Mazutis, J. Gilbert, W. L. Ung, D. A. Weitz, A. D. Griffiths and J. A. Heyman, Nat. Protoc. 2013, 8, 870.

[3] J. C. McDonald, D. C. Duffy, J. R. Anderson, D. T. Chiu, H. Wu, O. J. A. Schueller and G. M. Whitesides, *Electrophoresis* 2000, **21**, 27.

Fig S1: Bulk kinetics assay:

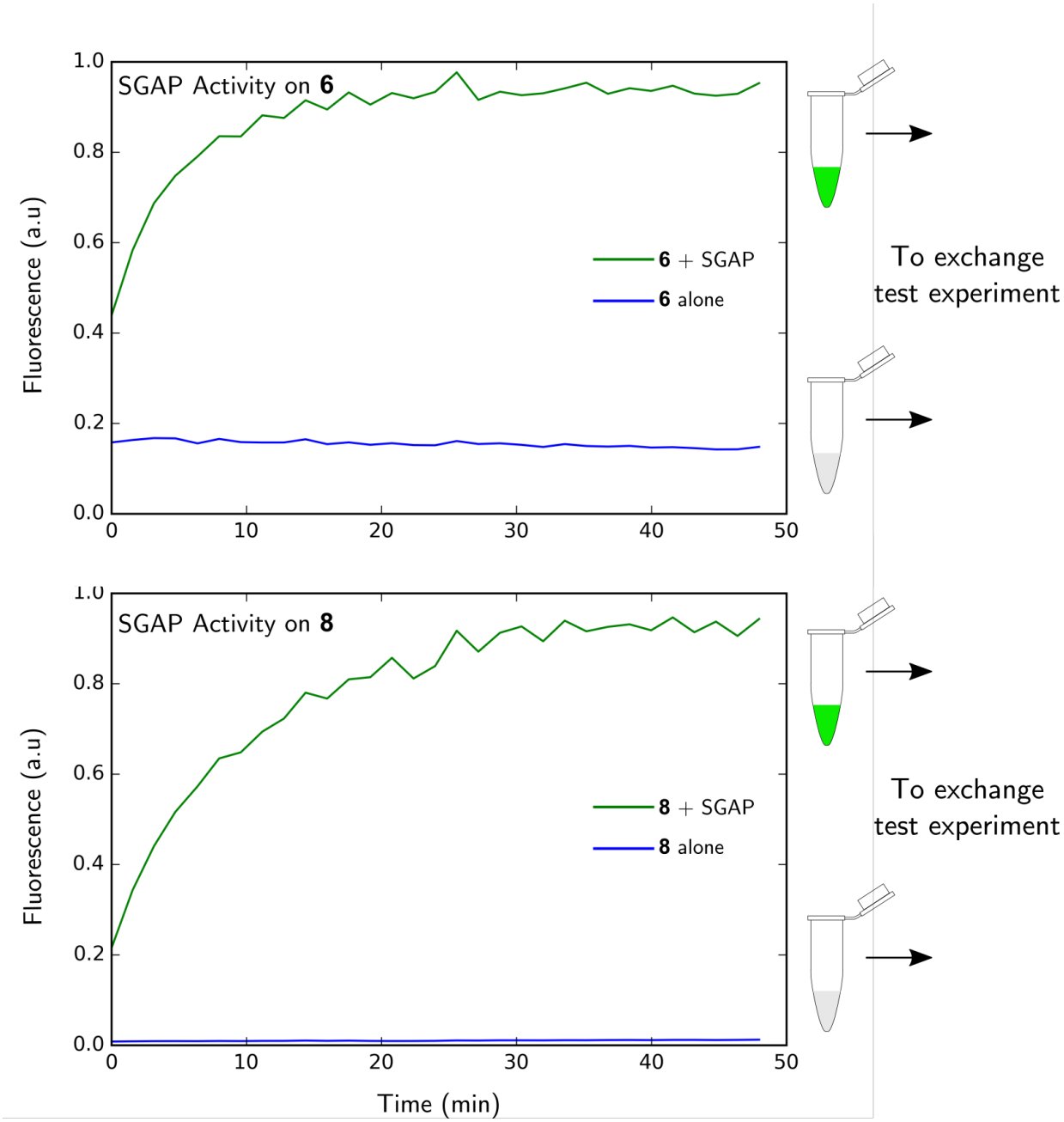


Fig. S1: Fluorescence evolution of **6** and **8** in the presence (green) or absence (blue) of SGAP enzyme.

For each substrate (**6** or **8**), once saturation of the fluorescence of the positive assay was achieved, well contents from positive and negative assays were retrieved into Eppendorf tubes. A control fluorescent dye (dy647, dyomics, ex: 653 em: 672 nm) that does not get exchanged between droplets was added to the positive and the negative solutions at a final concentration of respectively 500 nM and 1 µM. Once mixed, the solutions were used to perform exchange test experiments in droplets (Fig. S2).

Fig S2: Workflow of the exchange test experiments in droplets:

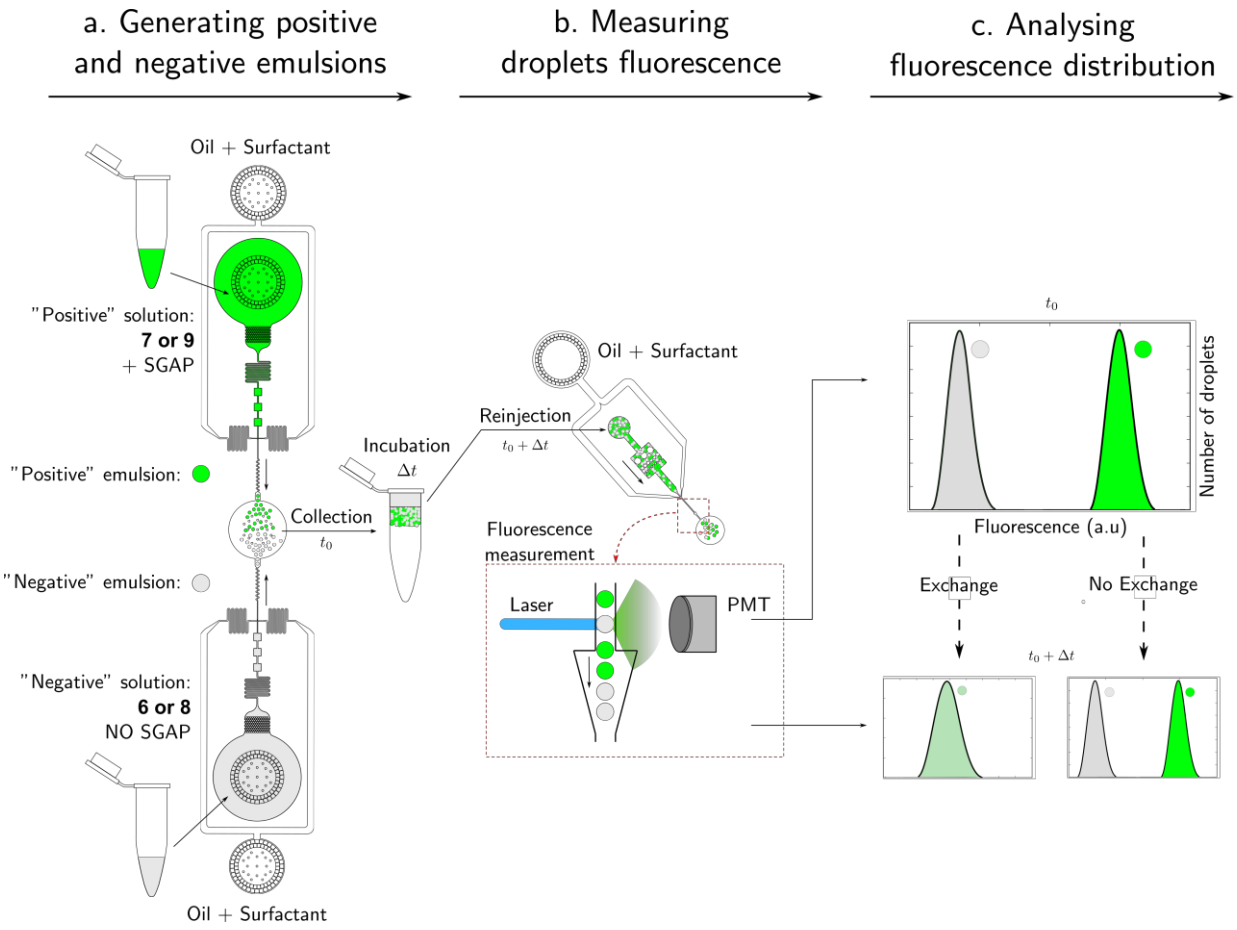


Fig. S2: Workflow of the exchange test experiment in droplets

a. Both positive (green, with SGAP) and negative (grey, without SGAP) solutions were compartmentalized separately in 20 pL droplets at a generation frequency of approximately 1kHz. They were collected simultaneously using a double droplet maker design (two droplet makers with a common outlet). We used HFE 7500 (3M) fluorinated oil containing "008-fluorosurfactant" (RAN biotechnologies) as surfactant (2% wt.). In each droplet maker, the oil phase was injected at 1 bar into the oil inlet of the chip, while aqueous phase was injected at 1.2 bar. Solutions were pressurised using Fluigent pressure-controlled pumps.

Collection was performed in an Eppendorf tube sealed with a plug of PDMS. The resulting emulsion was left to incubate at room temperature (23°C), in the dark.

b. The reinjection microfluidic chip was placed on the stage of an inverted fluorescence microscope (Nikon Eclipse Ti). Two monochromatic lasers (488 nm Coherent and 640 nm Crystalaser), and two photomultipliers (PMT, Hamamatsu) were directed to the microscope objective using a dichroic mirror and a dedicated set of filters to hit the microfluidic channel in which droplets were circulating and to measure their fluorescence. As the droplets crossed the laser beam, the PMT signal was acquired and recorded via an FPGA card (National Instruments) connected to a computer. The signal was then analysed by custom-developed software which measures and records the fluorescence intensity of each droplet, in two spectral windows: 506-545 (rhodamine fluorescence) and 671-746 (control fluorescence).

To assess exchange progression over time of the hydrolysed substrate, part of the emulsion was reinjected using pressure pumps (Fluigent) immediately after the end of collection (t_0) and after different incubation time points ($t_0 + \Delta t$). Oil and aqueous phases were pressurised at 0.5 mBar, 1 kHz reinjection frequency. During reinjection the fluorescence of each droplet was recorded. The fluorescence of the control dye is used to ensure a constant Z-focus of the microscope throughout the experiment. Fluorescence distributions in both spectral windows were then compared with t_0 fluorescence distributions. Reinjection and fluorescence measurements were performed at t_0 + 0.5 h, t_0 + 1 h, t_0 + 2 h, t_0 + 3 h, t_0 + 4 h and t_0 + 16 h (fluorescence distributions over time are depicted in Supporting Figure 3).

c. The evolution of the fluorescence intensity distribution can be analysed to assess the exchange rate of hydrolysed substrate between the emulsions. The two possible extreme cases are illustrated here. “No exchange”: negative + positive emulsion fluorescence distribution is bimodal and identical to t_0 distribution. “Exchange”: the distribution collapsed into one single mode at an intermediate intensity with respect to initial positive and negative emulsions. In the latter case, we can no longer distinguish positive and negative populations after the incubation time Δt. Therefore, in the case of a variant screening, it would be impossible to discriminate between low and high activity mutants of the SGAP enzyme.

Fig. S3: Exchange test experiment in droplets for compound 6:

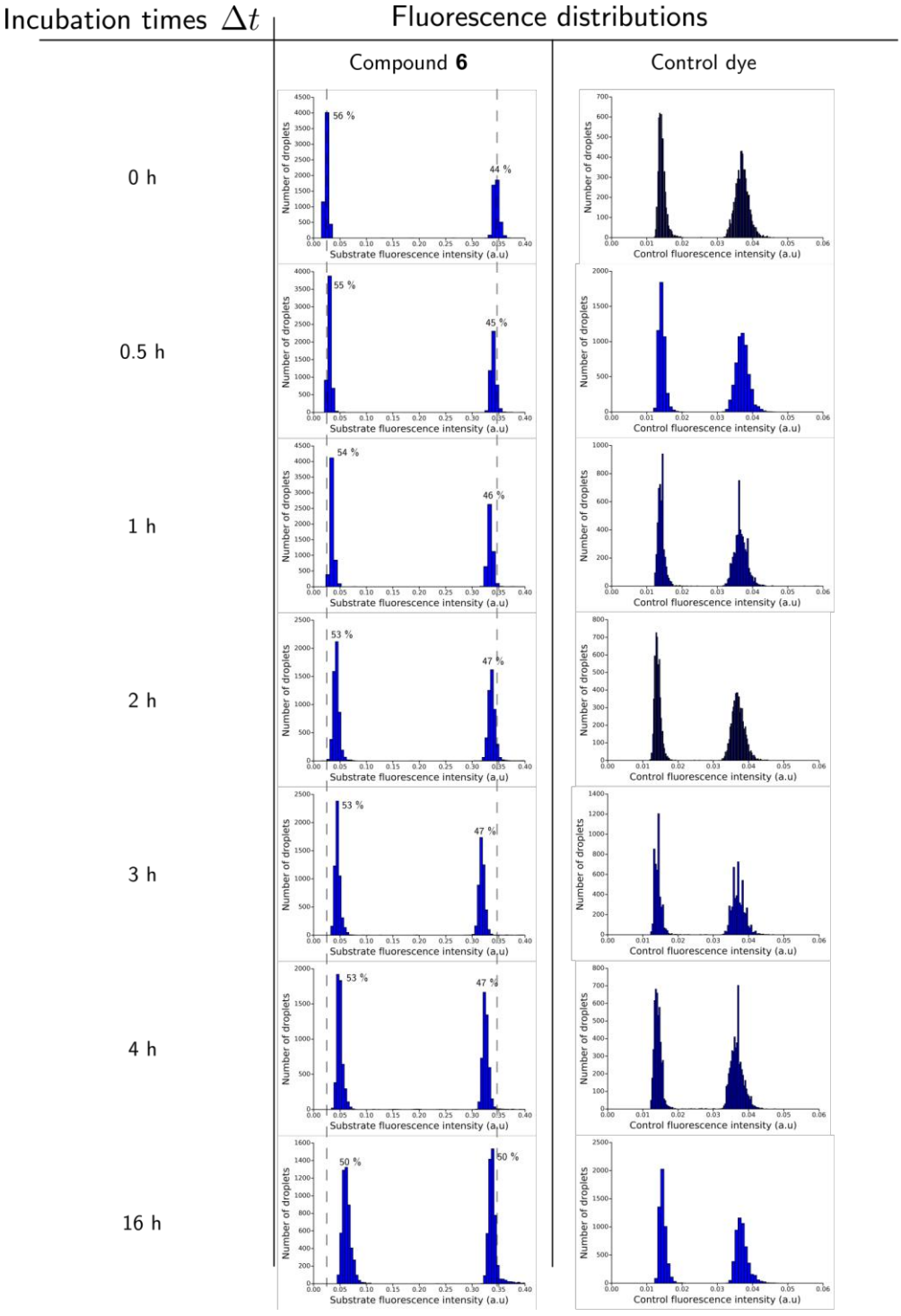


Fig. S3: Exchange test experiment for compound **6** and control dye.

The fluorescence profile (substrate fluorescence and control) of 10 000 droplets was measured at each time point. Minor changes in the droplet count ratio of each subpopulation are explained by the sampling fluctuations and possible local heterogeneities in the collection tube.

Fig. S4: Exchange test experiment in droplets for compound 8 :

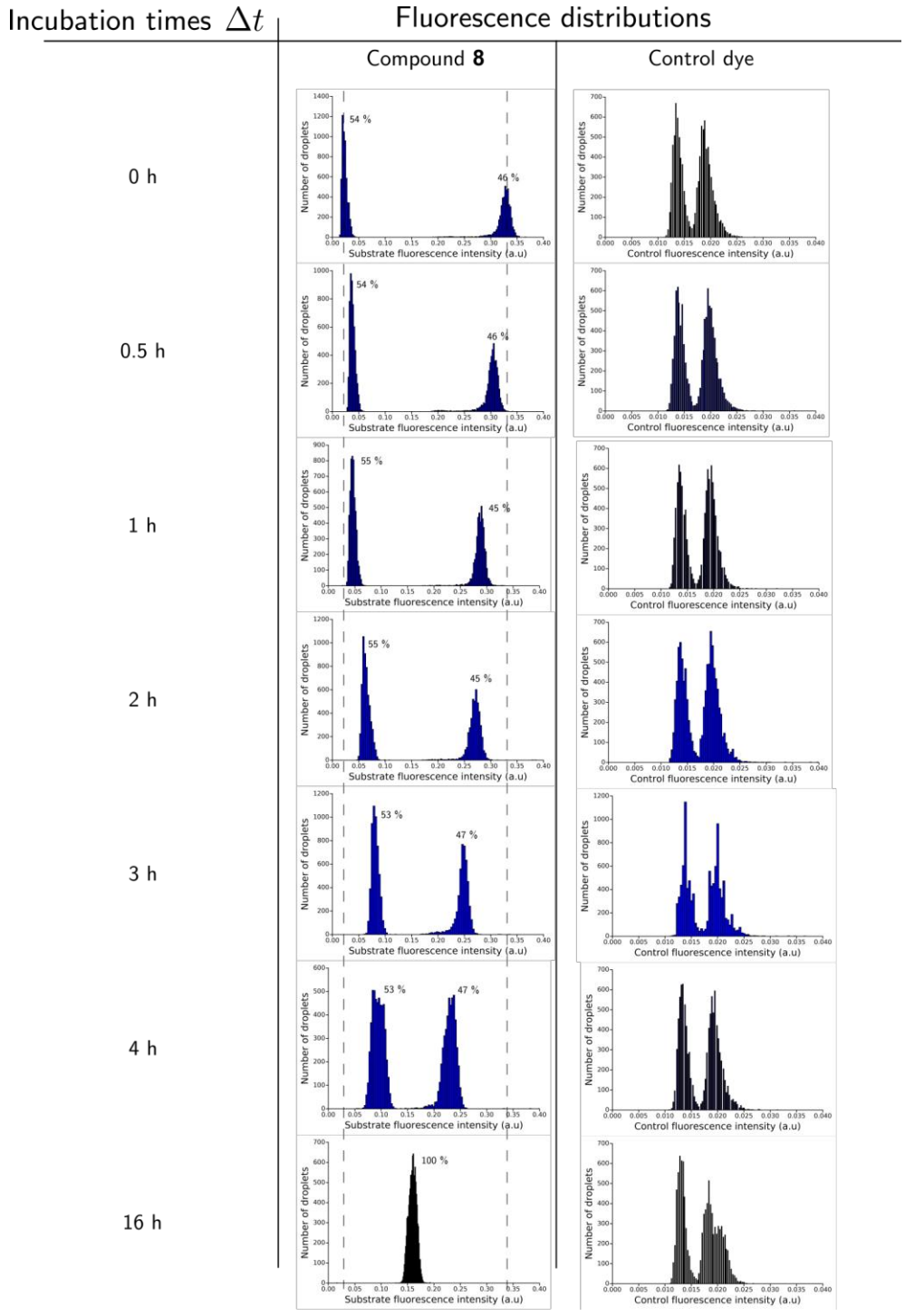


Fig. S4: Exchange test experiment for compound **8** and control dye.

The fluorescence profile (substrate fluorescence and control) of 10 000 droplets was measured at each time point. Minor changes in droplet counts ratio of each subpopulation are explained by the sampling fluctuations.

Fig. S5: Quantifying the exchange of probes 6 and 8:

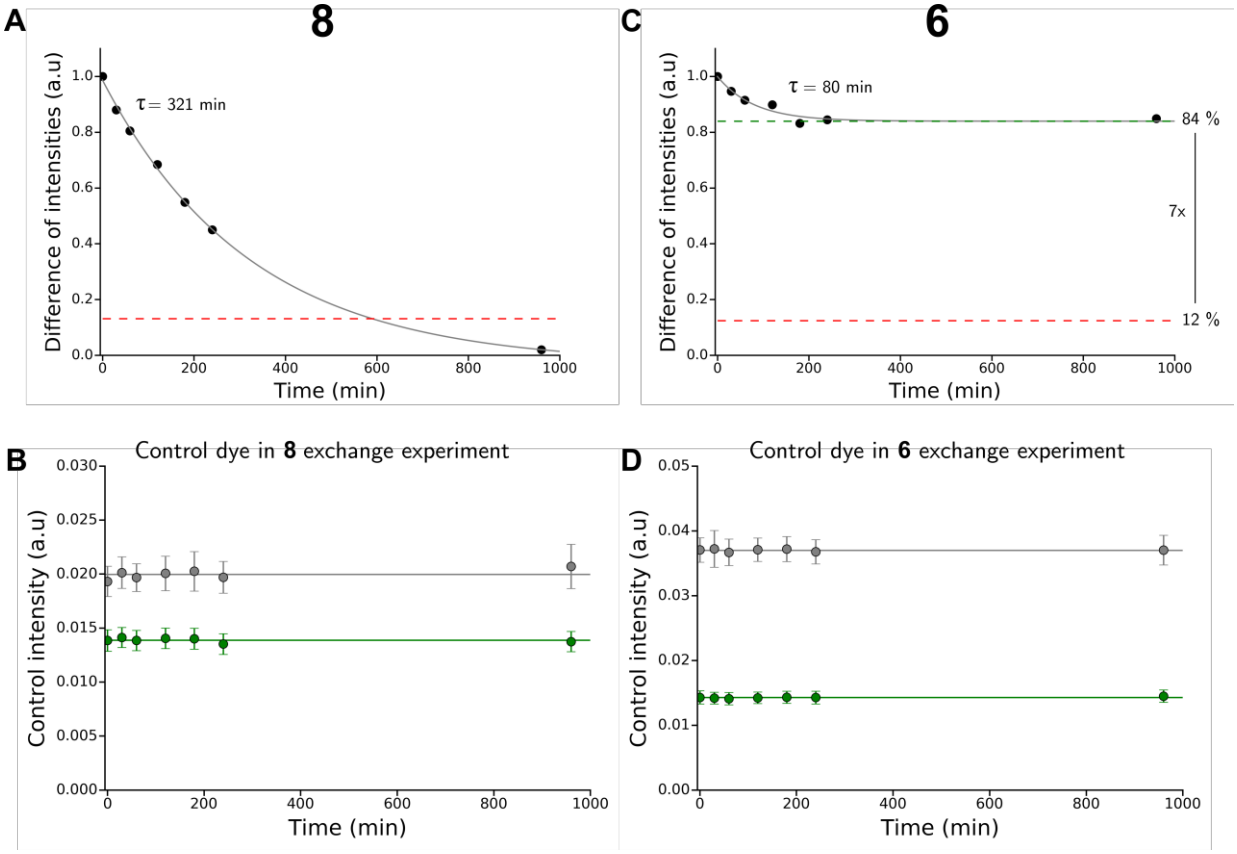


Fig. S5: Quantifying the exchange of **6** and **8**

A and **C**: For the compounds **6** and **8** respectively, the difference of the average fluorescence of positive and negative populations is computed at each time point and normalized to the initial difference. The red dotted line represents the sum of the standard deviations: $\sigma_{positive} + \sigma_{negative}$. **A**: For **6,** it is no longer possible to distinguish between the two populations after 600 min. Data were fitted according to exponential decay with a time constant of 320min+/-15min. **C**: For **8**, following an initial transient exchange (exponential decay fitted time constant of 80 min+/-25min), the difference between the average fluorescence of positive and negative emulsions remains constant at 84% of the initial contrast. The mechanism of the initial transient exchange remains to be investigated.

B and **D**: A control dye that emits in infrared and that does not get exchanged between droplets, dy647, is present in the same droplets as **6** or **8**. Its fluorescence intensity is monitored simultaneously to that of **6** or **8** to ensure constant optical settings throughout the experiment. **B:** Control intensity during the **8** exchange experiment. **D:** Control intensity during the **6** exchange experiment.

Fig. S6: Testing SGAP activity towards 6 in droplets - microfluidic chip design:

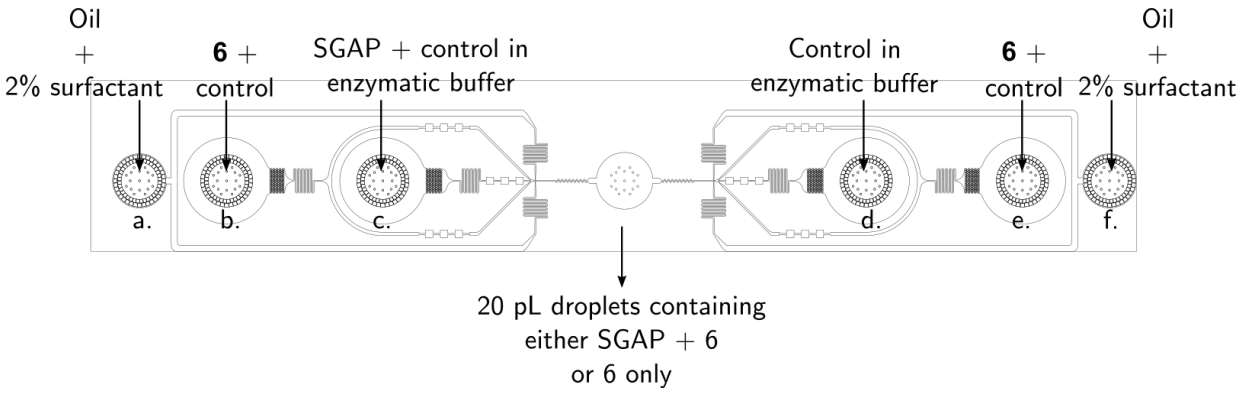


Fig. S6: Testing SGAP activity towards 6 in droplets

For the purpose of this experiment, pluronic F-127 (Sigma Aldrich) was added to the enzymatic buffer (0.4% w/v). A solution of **6** and control dye "dy647" was prepared in enzymatic buffer to the respective final concentrations 100 µM and 10 µM and connected to the b. and e. inlet. A solution of SGAP enzyme and control dye "dy647" was prepared in enzymatic buffer to the respective final concentrations 0.2 mg/mL and 10 µM and connected to the c. inlet. Finally a solution of control "dye647" was prepared in enzymatic buffer to 10 µM final concentration and connected to the d. inlet. Except for the presence of two aqueous inlets in this experiment, everything was performed according to the exchange test experiments protocol. The droplets fluorescence was measured in the same spectral windows, during production (t_0) and after 16h of incubation at room temperature (23°C) in the dark during reinjection (same device as in Fig. S2). The control fluorescence was used to ensure comparable measurements between t_0 and t_0 + 16h.

Fig. S7: Signal to noise ratio at different concentrations of 6:

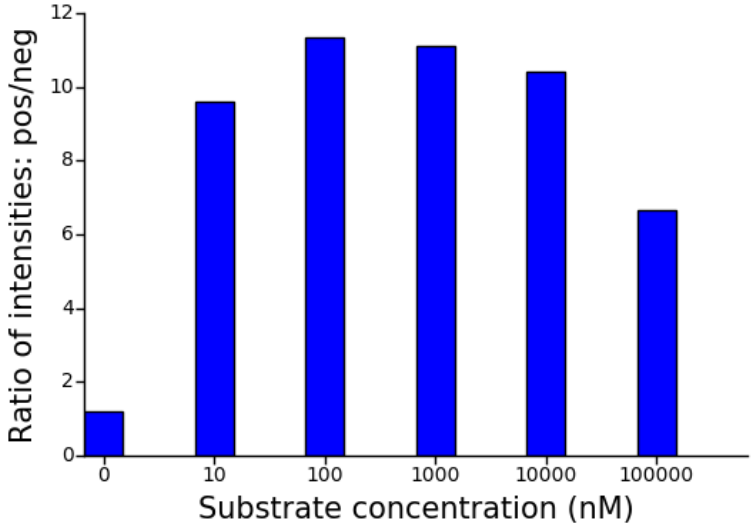


Fig. S7: Signal to noise ratio as different concentrations of 6

To investigate the influence of substrate concentration on signal to noise ratio, enzymatic assays were performed with the following concentrations in enzymatic buffer supplemented with pluronic F-127, 0.4%, in a spectrophotometer (100 µL per assay):

Assay	SGAP final concentration (mg/mL)	**6** concentration (nM)
1	0.01	0
2	0.01	10
3	0.01	100
4	0.01	10^3
5	0.01	10^4
6	0.1	10^5

In each case, a negative control (no enzyme) was also performed. For each concentration, once hydrolysis was complete, the ratio between the final positive assay fluorescence and the final negative assay fluorescence was computed. An optimum is found between 100 nM and 1 µM where the positive assay is 11 times more fluorescent than the negative control. Between 10 µM and 100 µM this ratio is located between 10 and 7, which is consistent with the results of our experiments in droplets.

Fig. S8: Discrimination of different enzymatic activity levels:

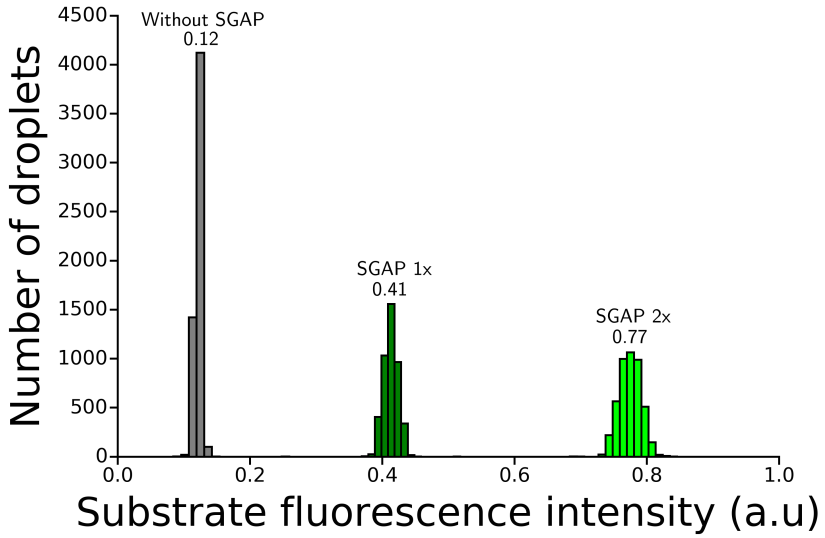


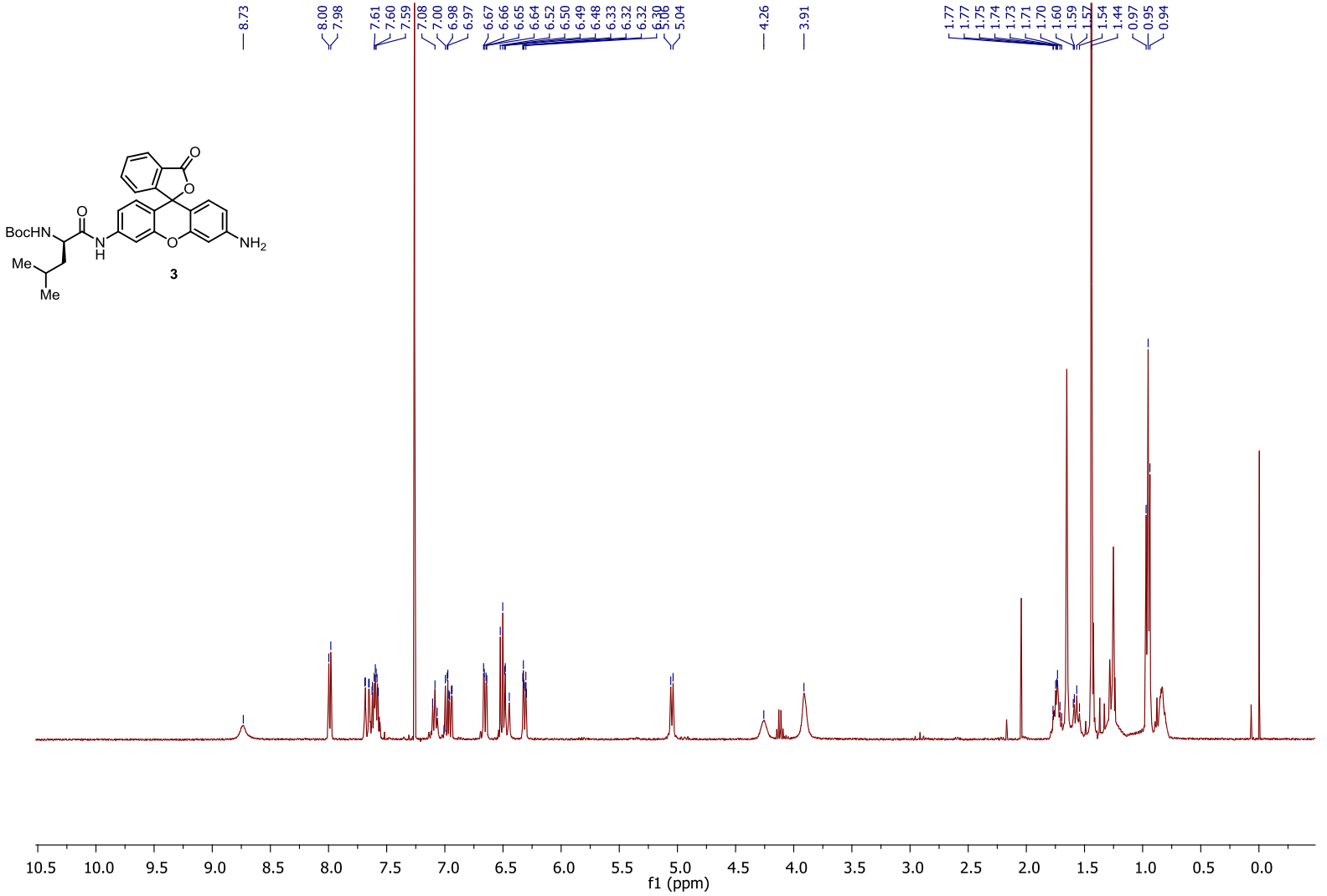
Fig S8: Discrimination of different enzymatic activity levels using our droplet microfluidics assay.

We have applied our assay to droplets containing SGAP at three different concentrations, mimicking enzymes with different k_{cat} Michaelis Menten parameters. We produced three emulsions containing probe **6** and either no SGAP, SGAP at 0.1 mg/mL (1x), or SGAP at 0.2 mg/mL (2x). The fluorescence distributions of the three droplet populations after 30 s of incubation are plotted here. The three populations are very well discriminated, which would make sorting for either activity level straightforward. The average fluorescence of the 0.2 mg/mL SGAP emulsion (0.77 A.U.) is approximately twice as high as the fluorescence of the 0.1 mg/mL SGAP emulsion (0.41 A.U.), confirming that our droplet assay based on probe **6** allows to quantitatively measure relative reaction rates in droplets.

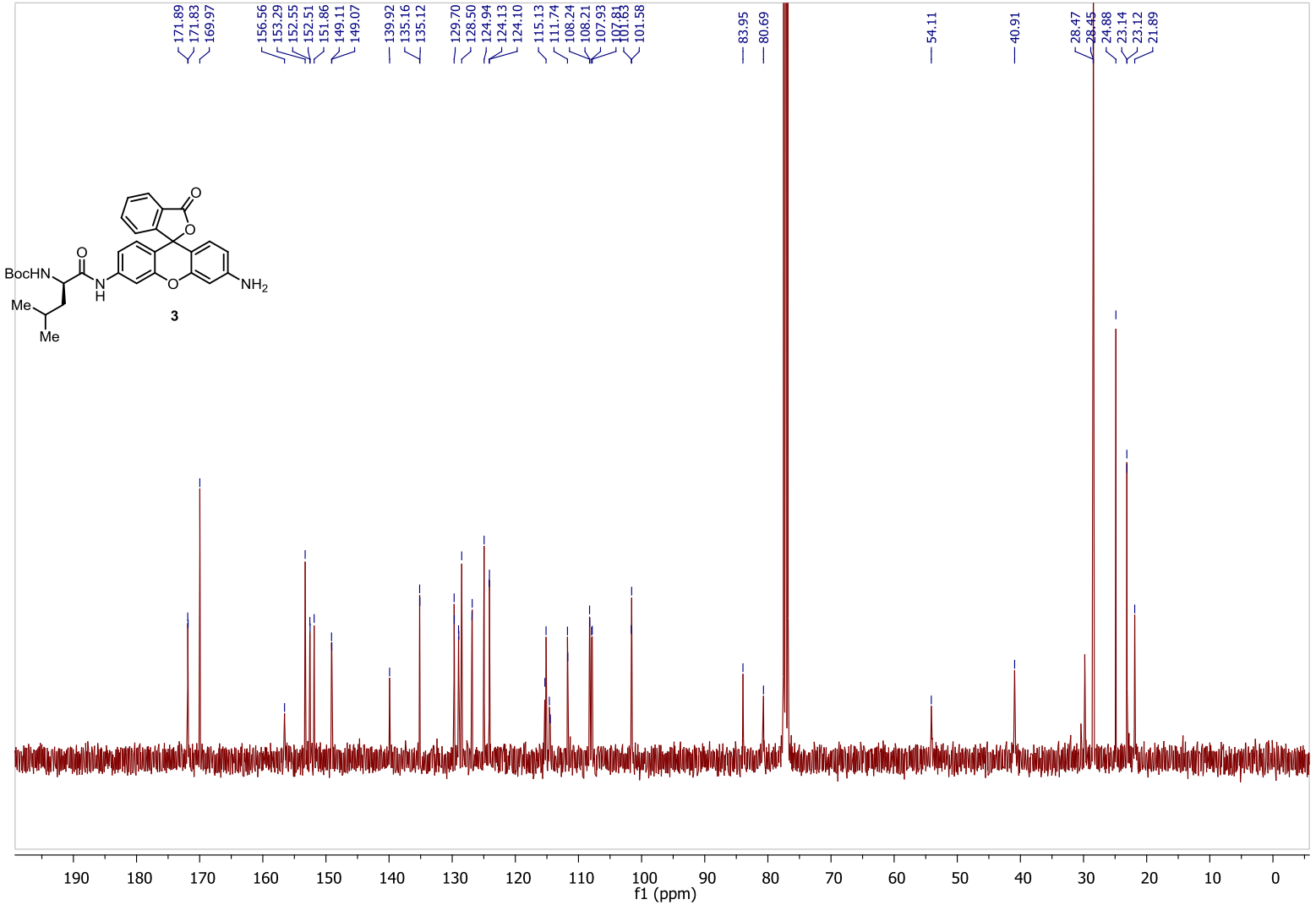
Protocol : This experiment was performed in a co-flow device similar to the design depicted in Fig. S6, but with three connected droplet makers instead of two and a second outlet to split the flow at the end of the device. Three SGAP solutions (0, 0.1 and 0.2 mg/mL) in enzymatic buffer supplemented with pluronic F-127 (final concentration, 0.4 % w/v) were encapsulated with a solution of probe **6** (100 µM in enzymatic buffer) using syringe pumps (Nemesys, aqueous phases: 50 µL/h, oil phases: 100 µL/h). Once the production was stable, the first outlet was directly connected to a reinjection chip using a piece of PTFE tubing (length: 5 cm, internal diameter: 0.33 mm). The time for the droplets to cross the tubing (time of incubation) was assessed to be approximately 30 s. During a mutant enzyme screening experiment, the incubation time should be optimized to cover the desired range of enzymatic activity levels. The substrate fluorescence of the droplets was recorded in the reinjection chip. A control dye was used to monitor the substrate concentration in all three populations (not shown here).

Copy of ^{1}H and ^{13}C spectra :

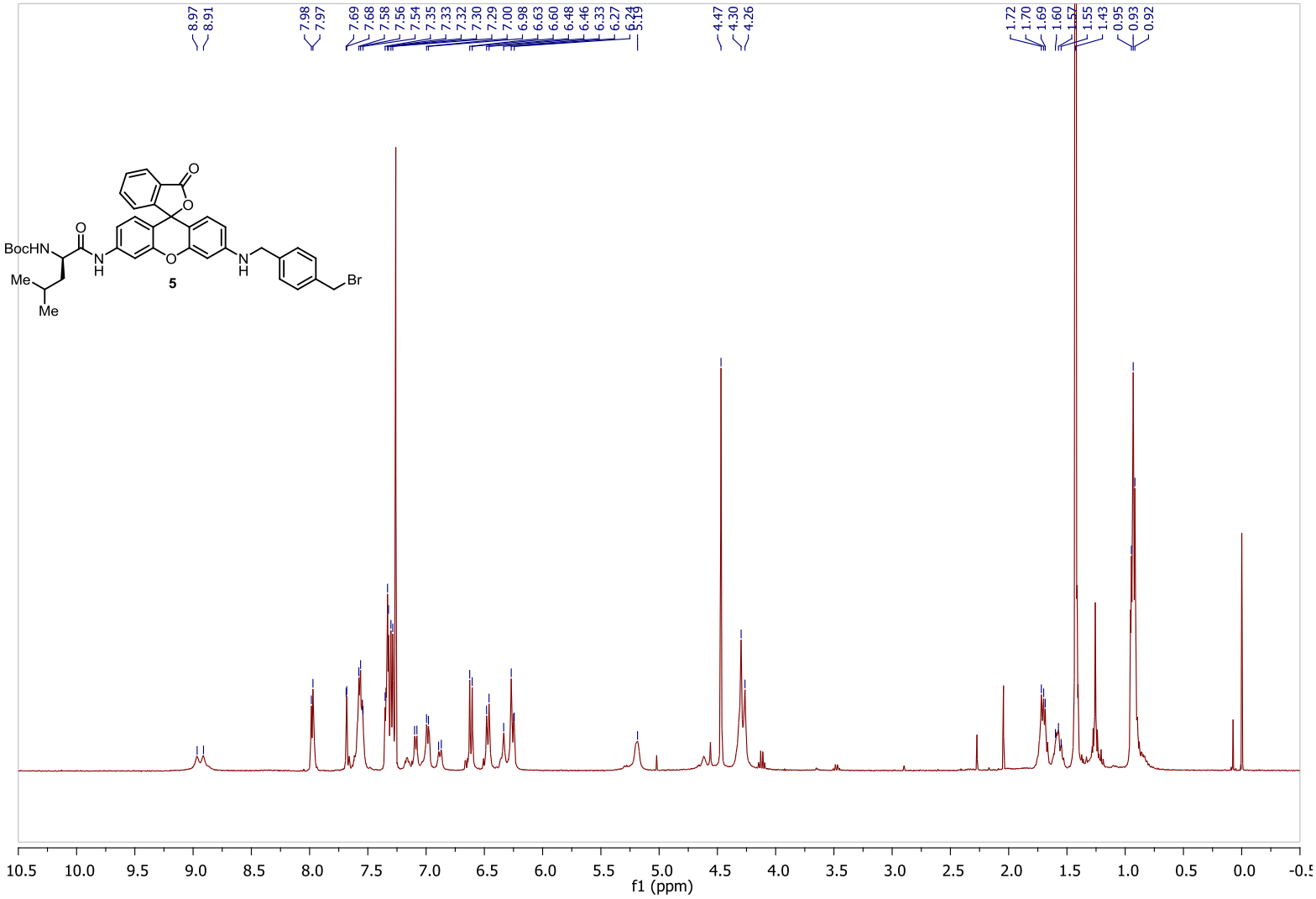
Copy of ^{1}H NMR spectra of **3**:



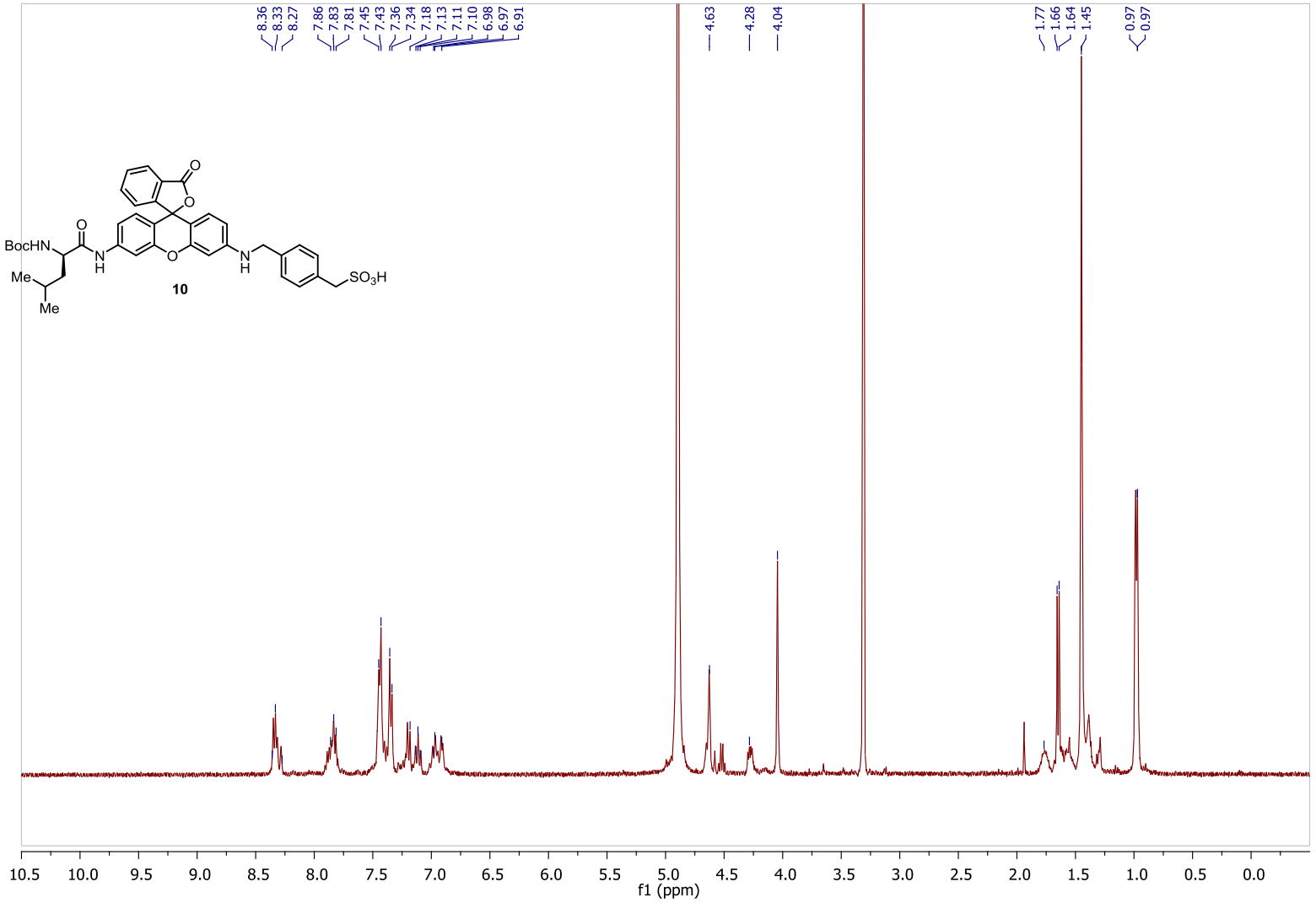
Copy of ^{13}C NMR spectra of **3**:



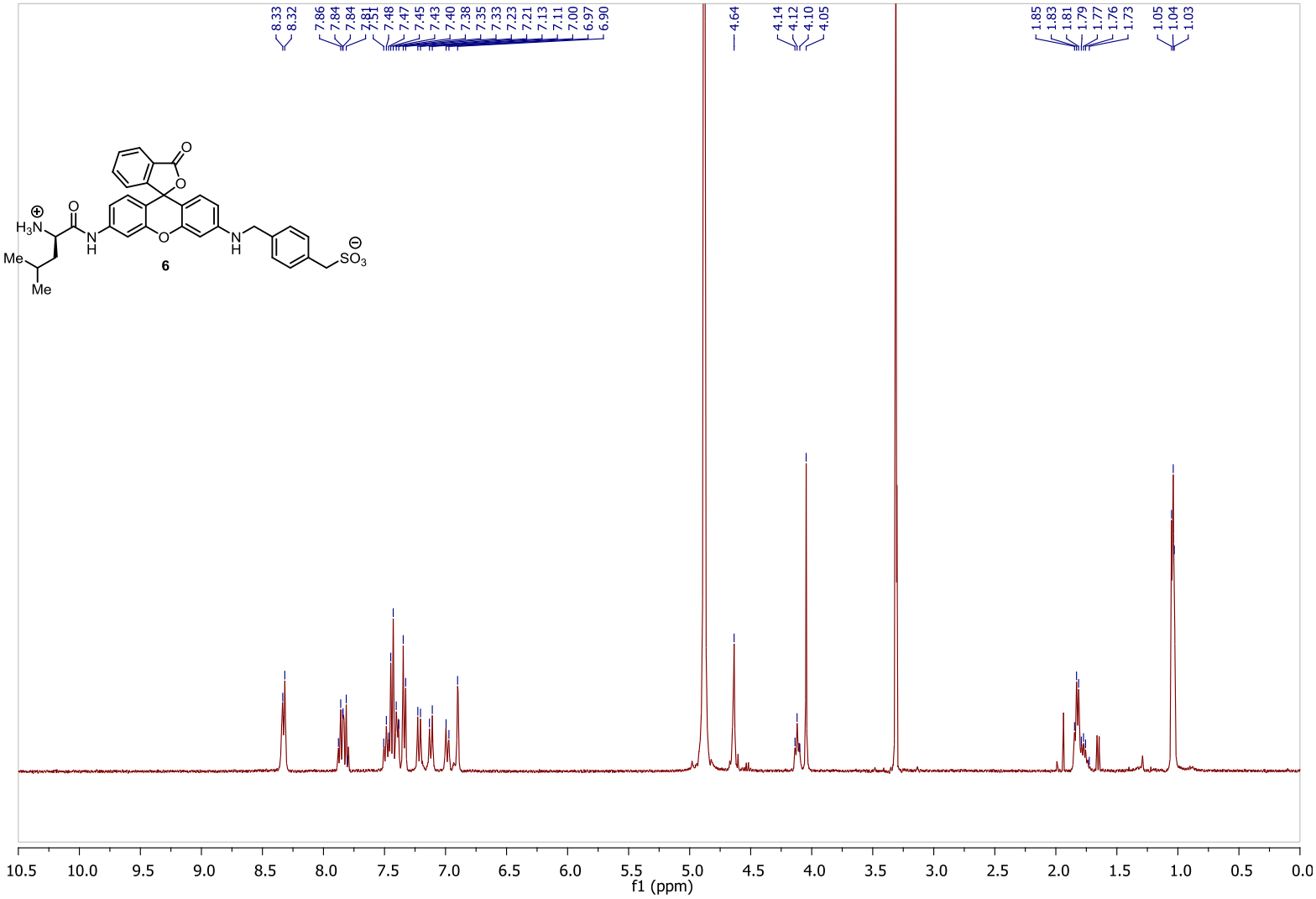
Copy of ^{1}H NMR spectra of **5**:



Copy of ^{1}H NMR spectra of **10**:



Copy of ^{1}H NMR spectra of **6**:



Copy of ^{13}C NMR spectra of **6**: