Electronic Supplementary Information

Investigation of tetrazine reactivity towards C-nucleophiles: pyrazolone-based modification of biomolecules

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1) Screening of potential carbanions/enolizable species in PBS 7.4 by fluorescence spectroscopy

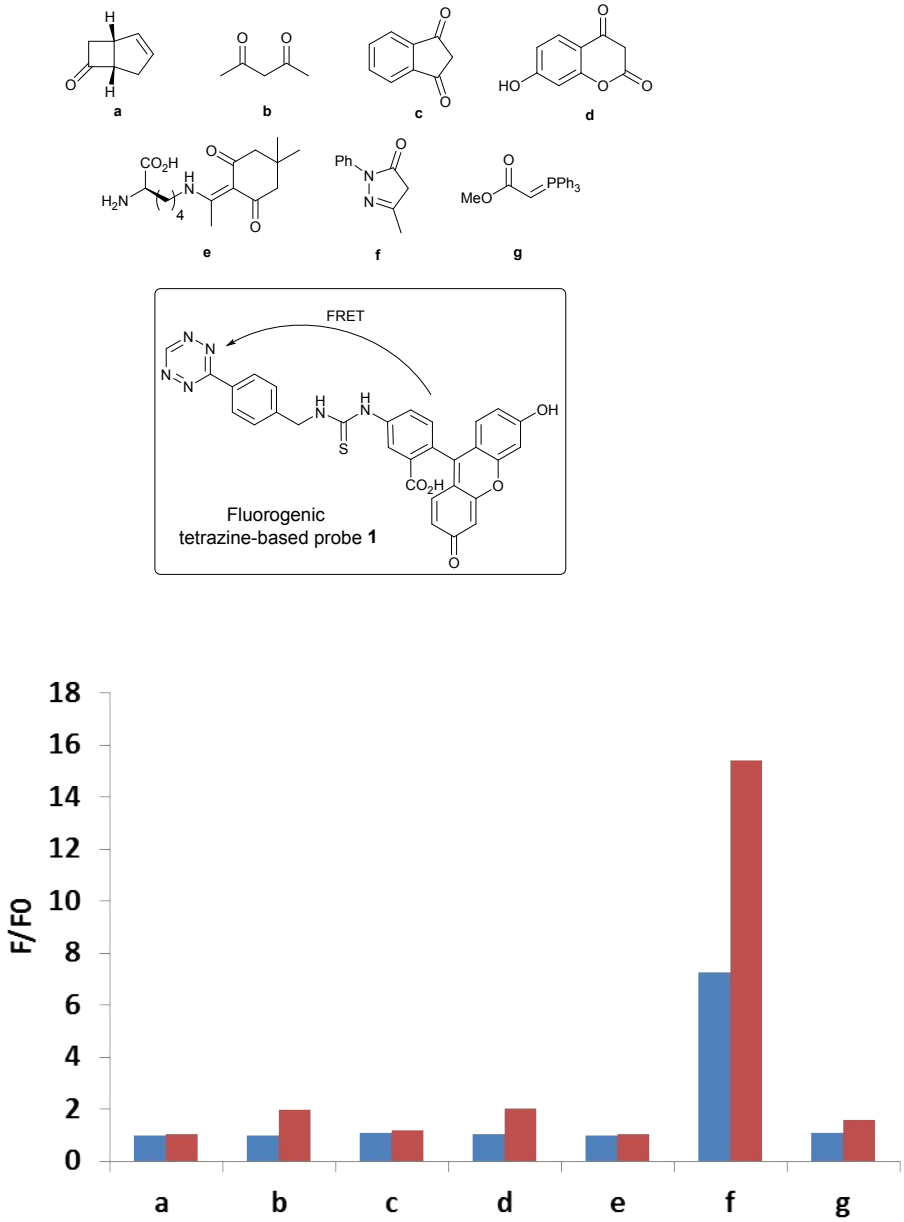
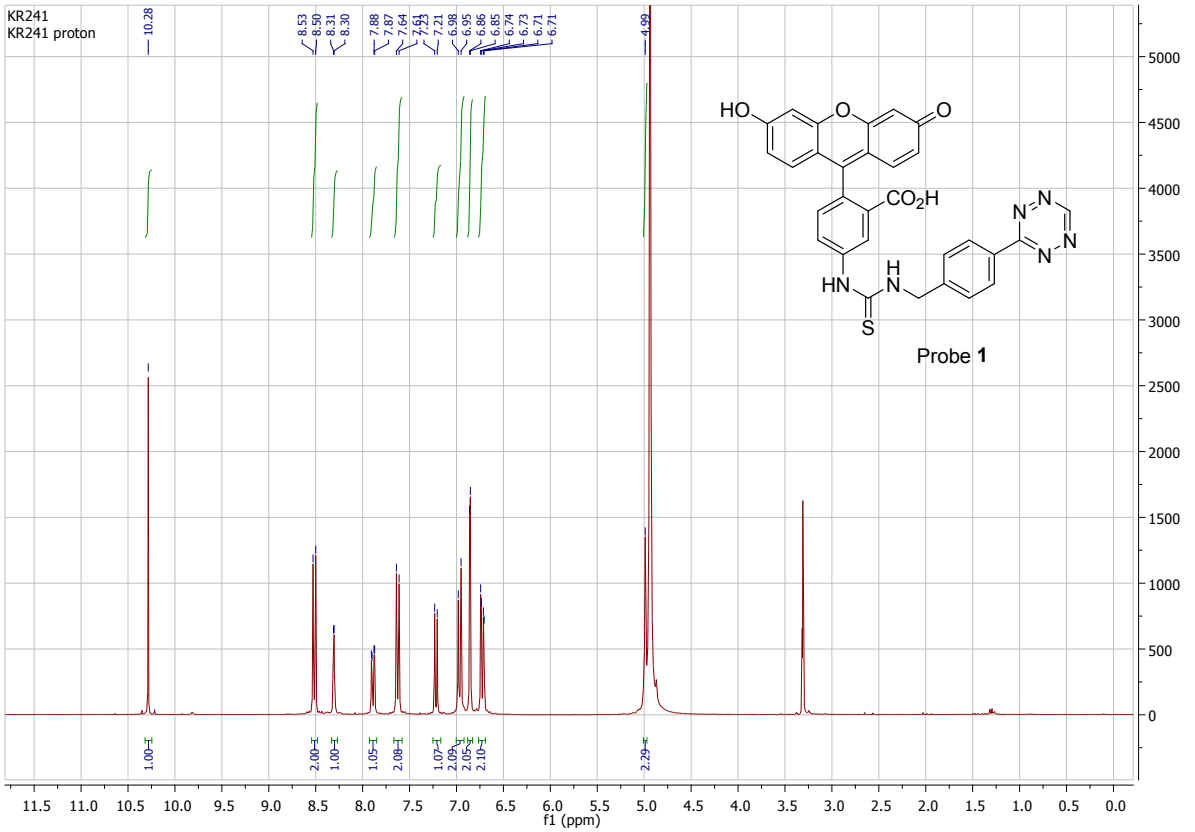
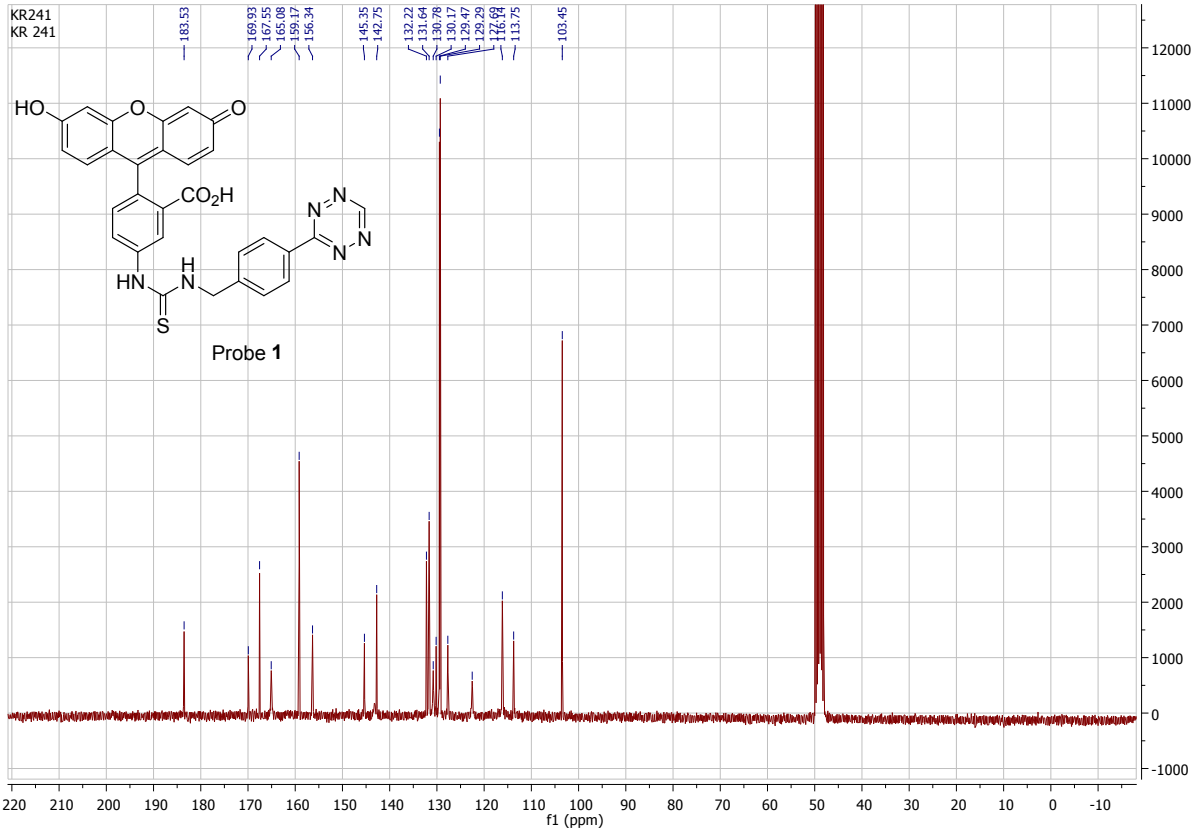


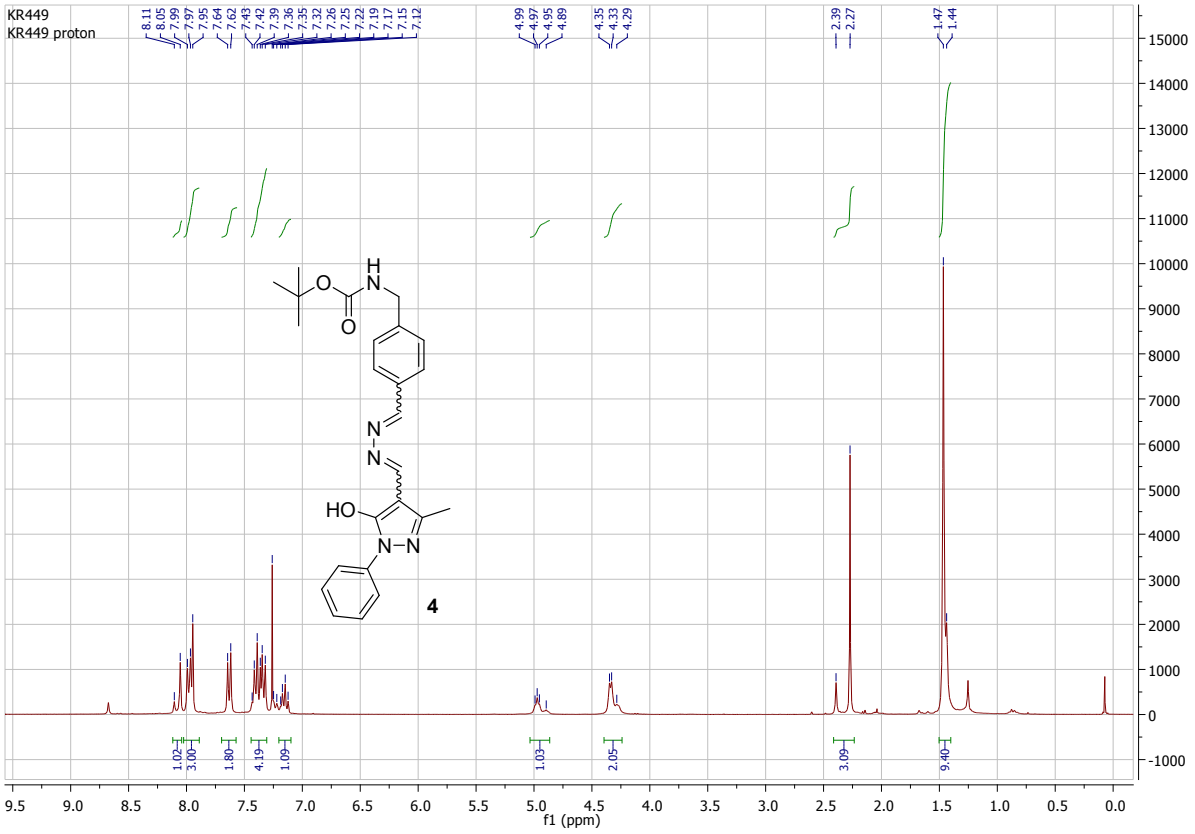
Figure S1: Screening study: fluorescence responses of tetrazine-based probe **1** (2.5 mM) at 2 (blue) and 60 min (red) after the addition of carbanions/enolizable species (10 mM, 4 equiv.) in PBS 7.4 at 25 °C at λ_{ex} 470 nm and λ_{em} 520 nm.

2) Characterization of compounds

2.1) Copies of ^{1}H, ^{13}C NMR spectra for compounds 1, 4-6, 8 and intermediates



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7.99
7.97
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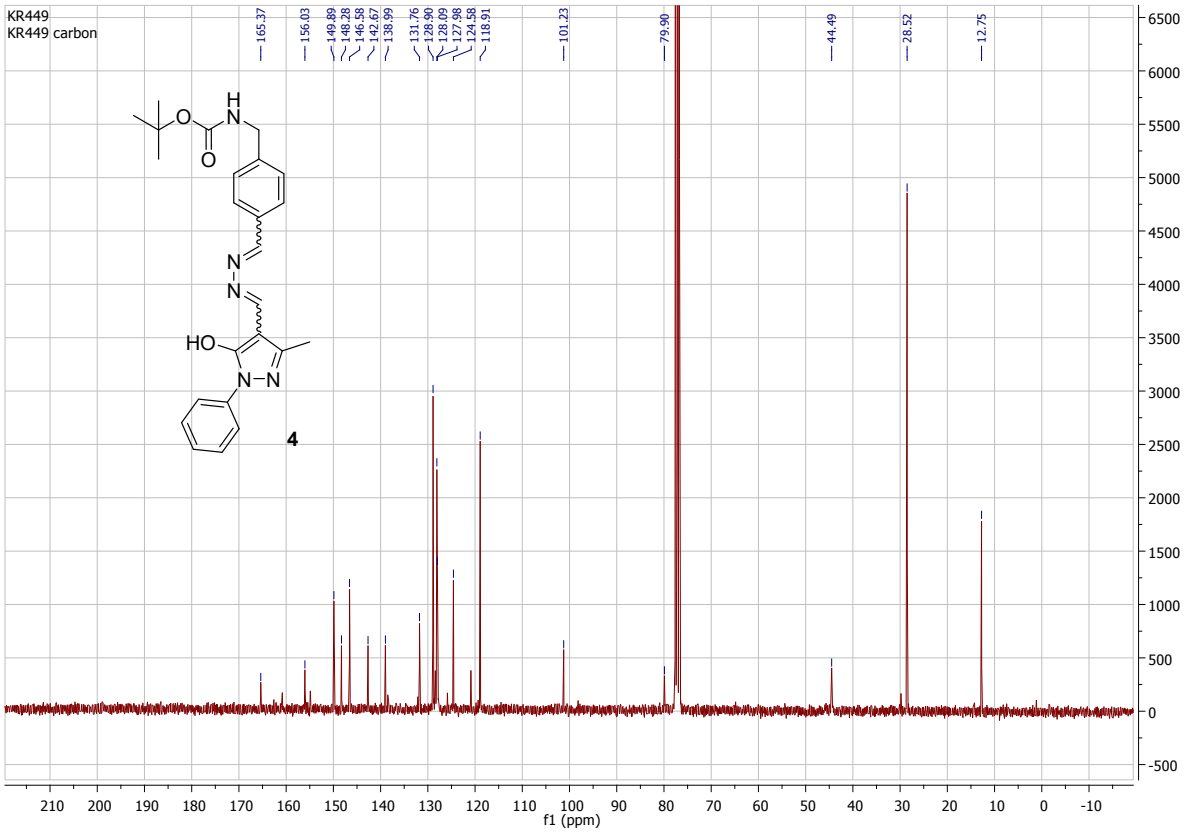
7.35
7.32
7.26
7.25
7.22
7.19
7.17
7.15
7.12
4.99
4.97
4.95
4.89
4.35
4.33
4.29
2.39
2.27
1.47
1.44
1.02
3.00
1.80
4.19
1.09
1.03
2.05
3.09
9.40
HO
N–N
4
f1 (ppm)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79.90
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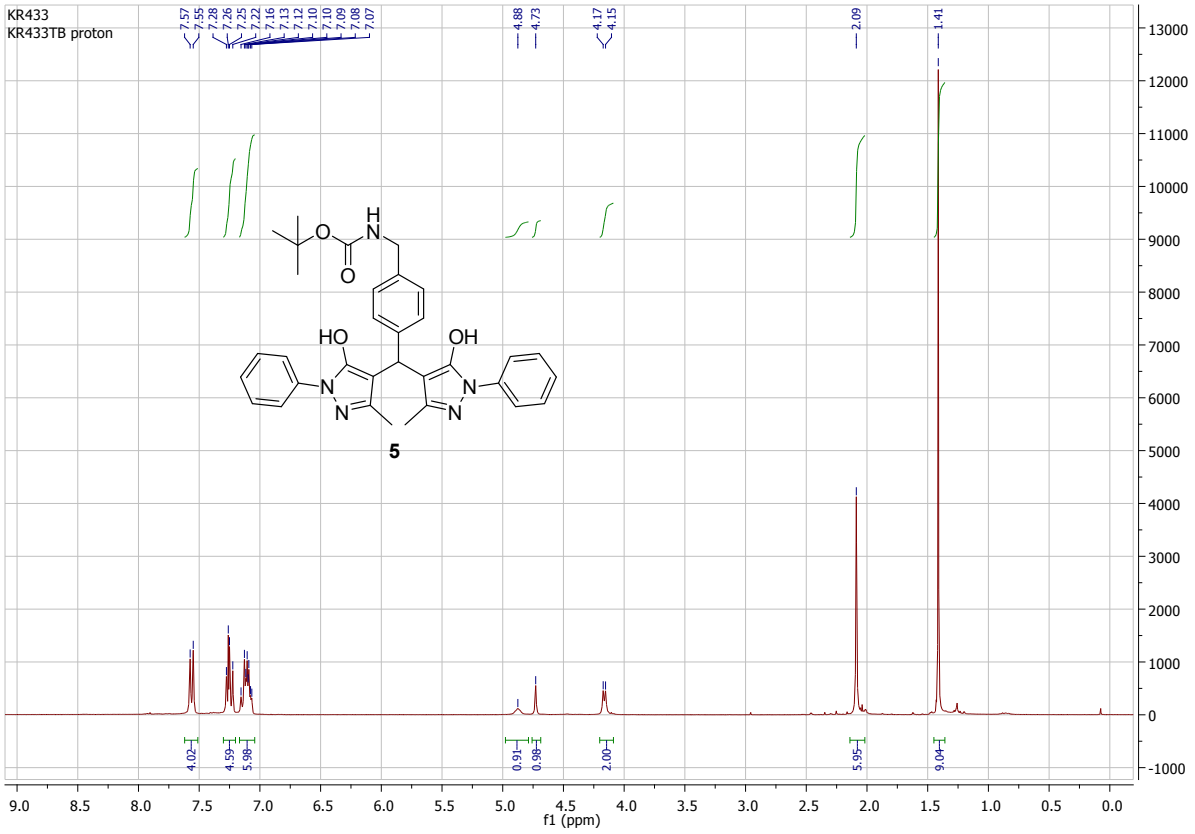
N–N
4
f1 (ppm)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7.10
7.09
7.08
7.07
4.88
4.73
4.17
4.15
2.09
1.41
HO
OH
N
N
N
N
O
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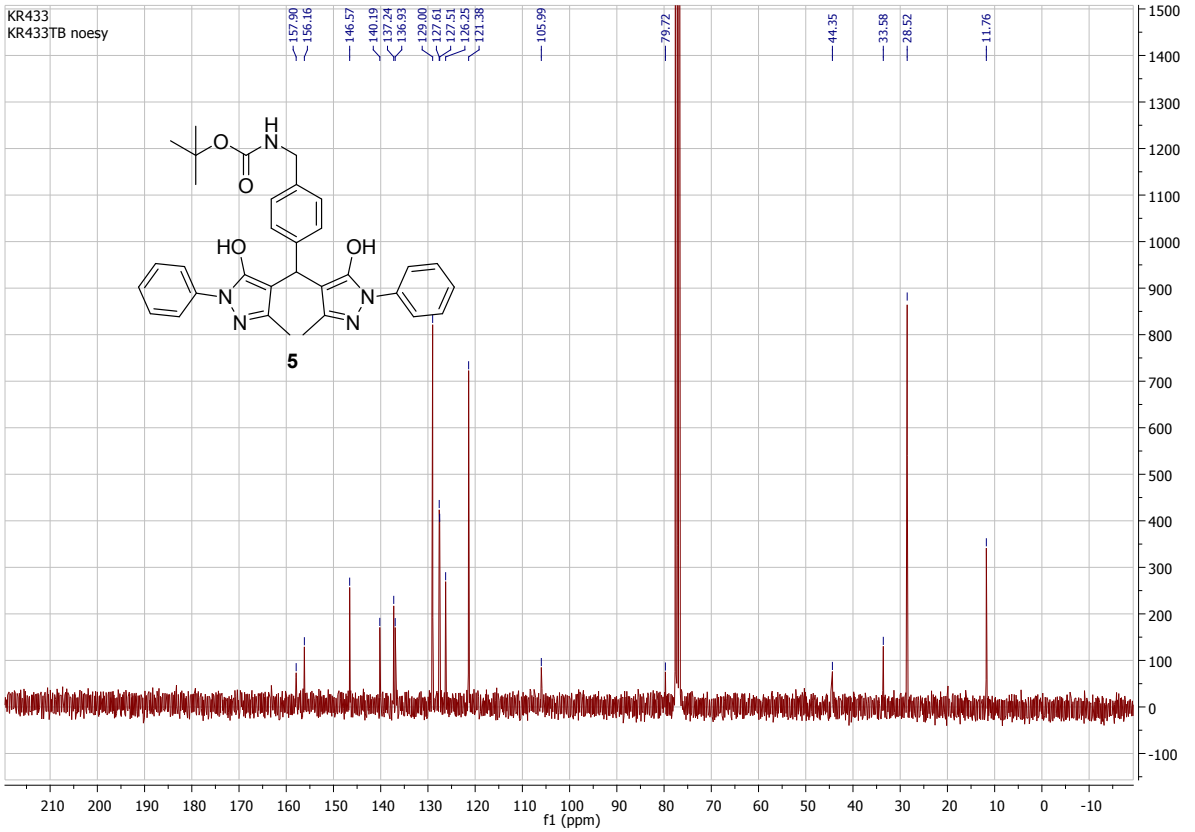
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105.99
79.72
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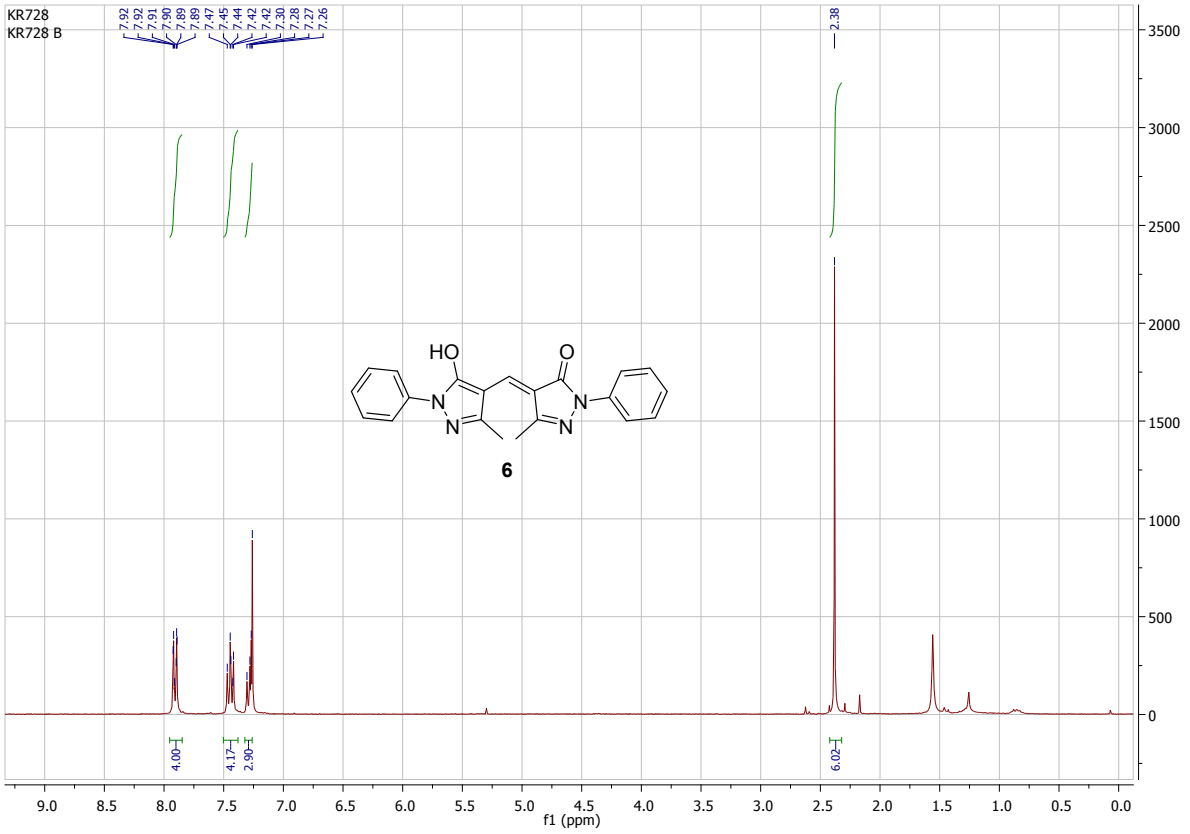
N
O
O
H
N
5
f1 (ppm)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KR728 B
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7.45
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7.42
7.30
7.28
7.27
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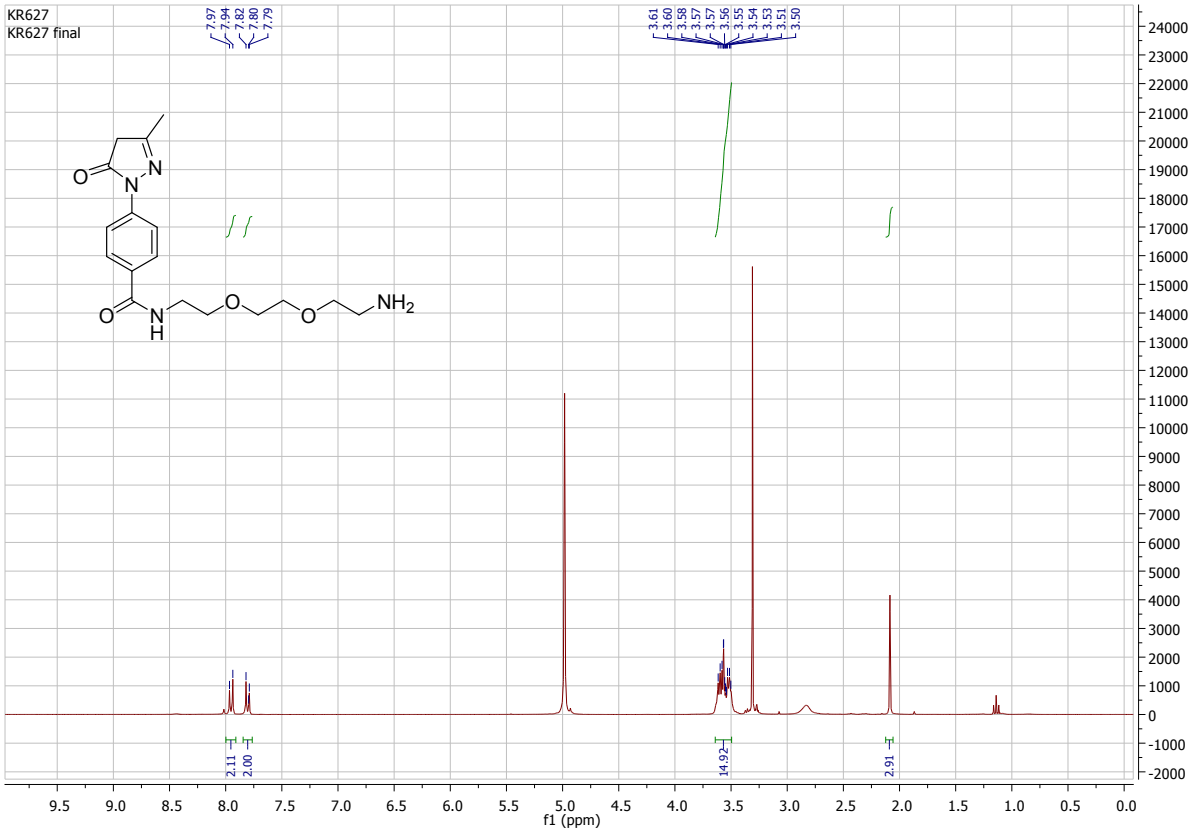
6
4.00
4.17
2.90
6.02
f1 (ppm)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KR627 final
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3.55
3.54
3.53
3.51
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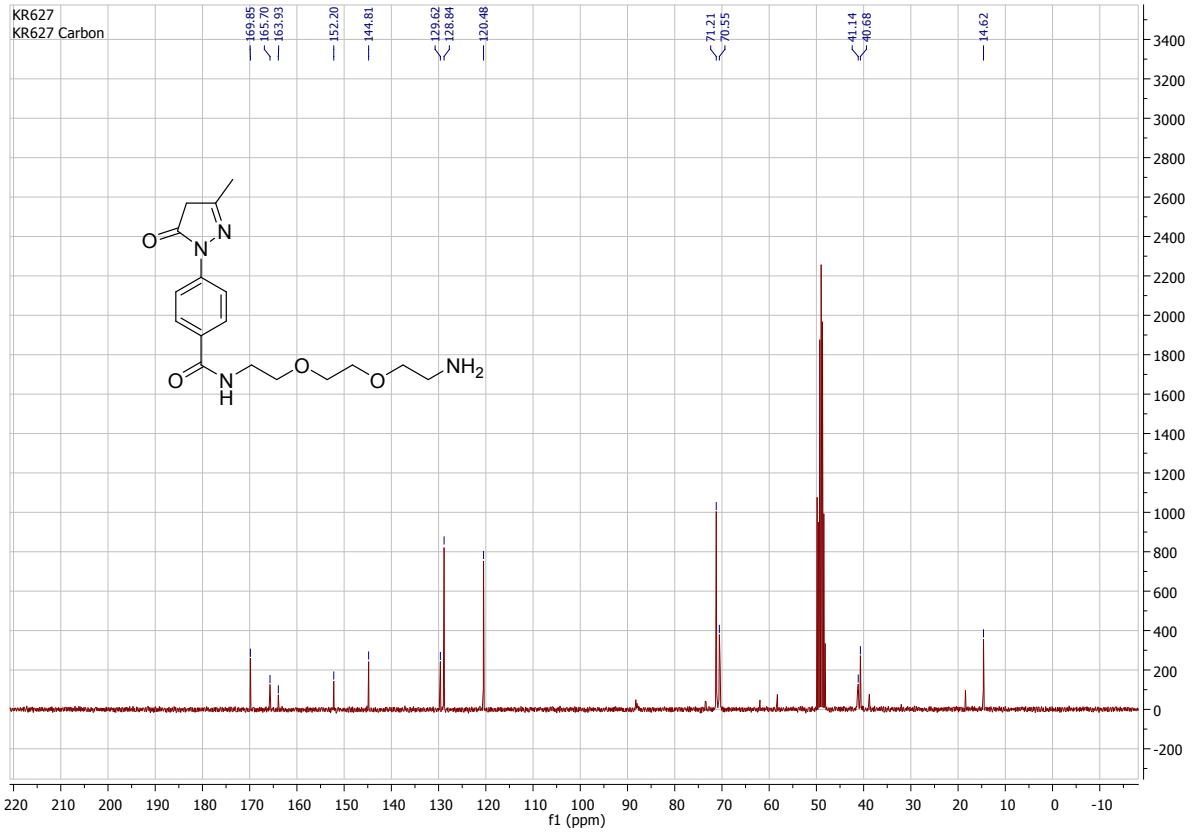
2.00
14.92
2.91
f1 (ppm)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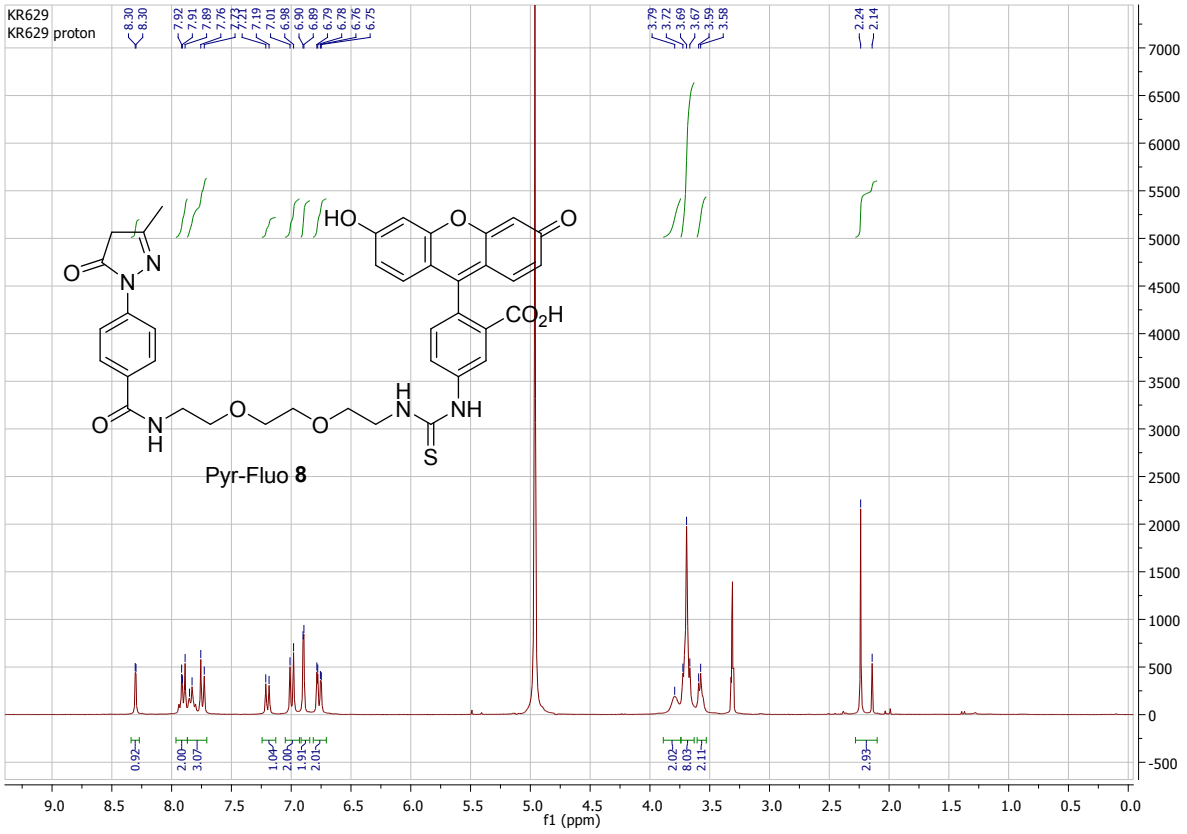
O
N
H
O
O
NH2
f1 (ppm)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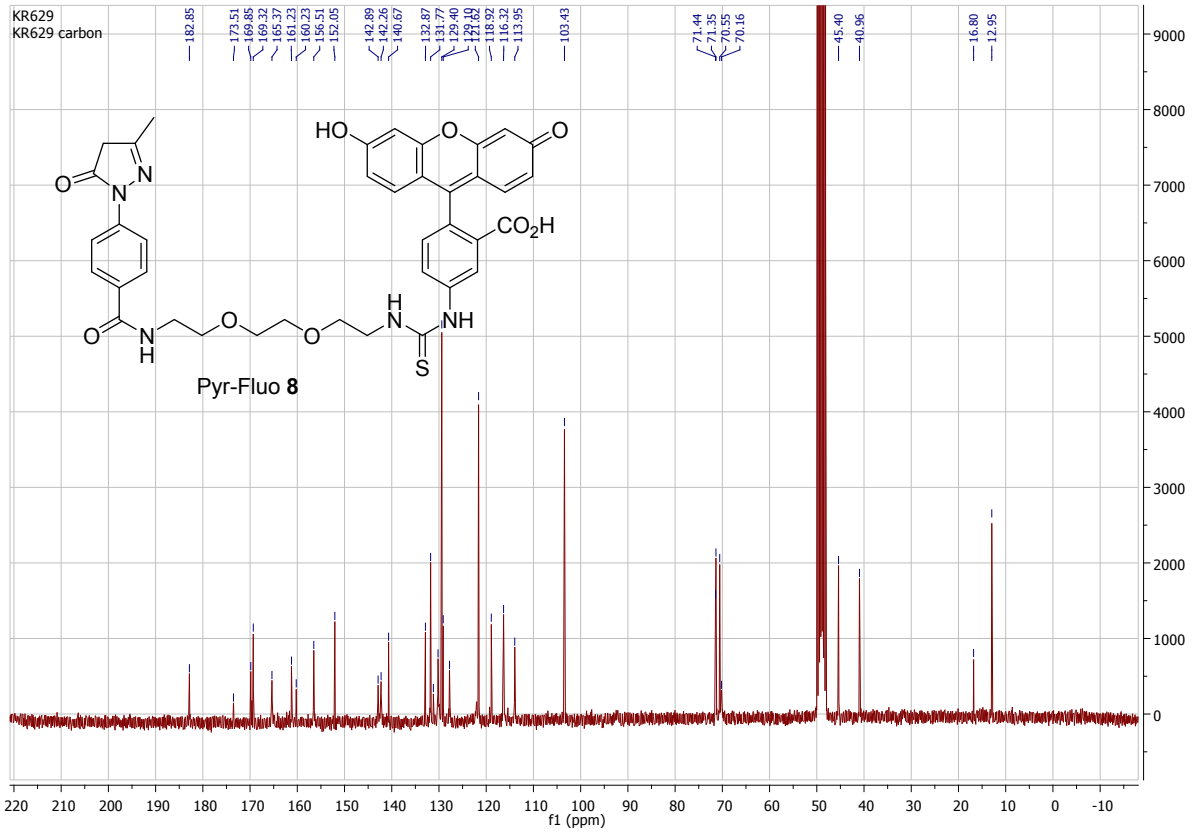
Pyr-Fluo 8
f1 (ppm)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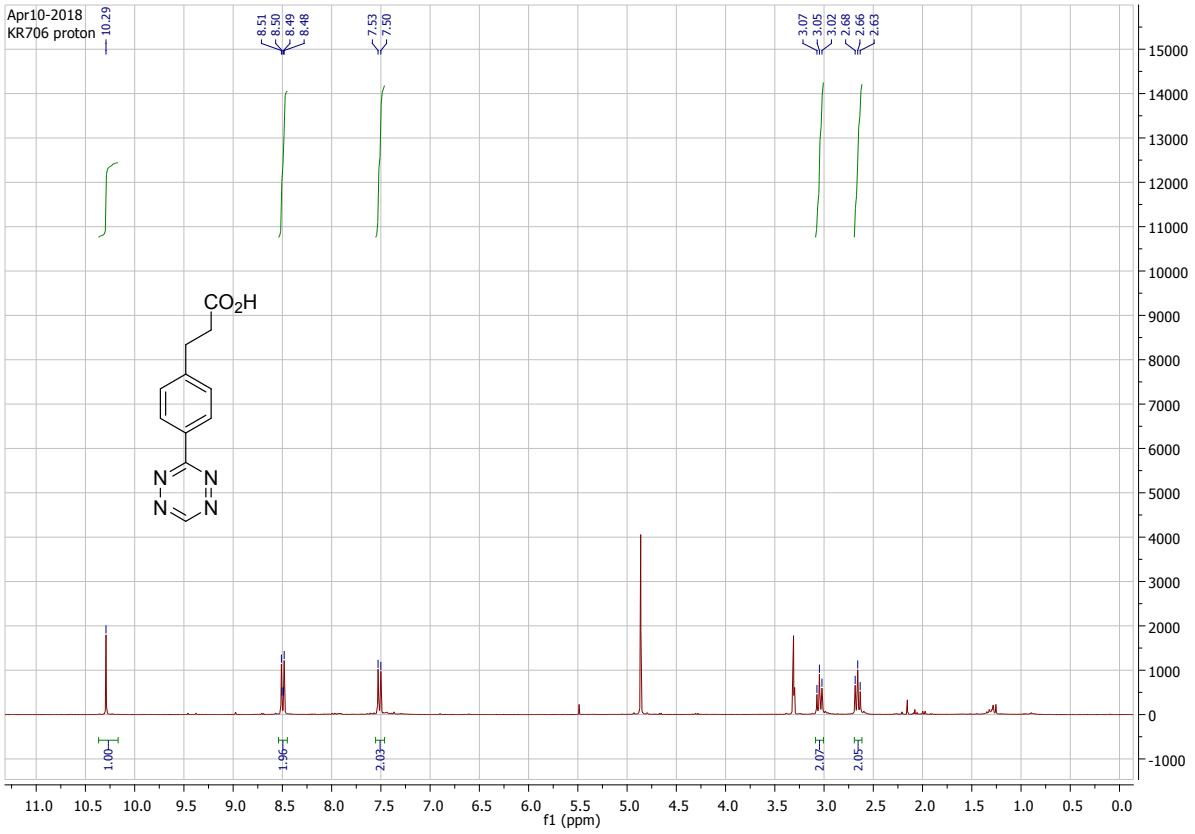
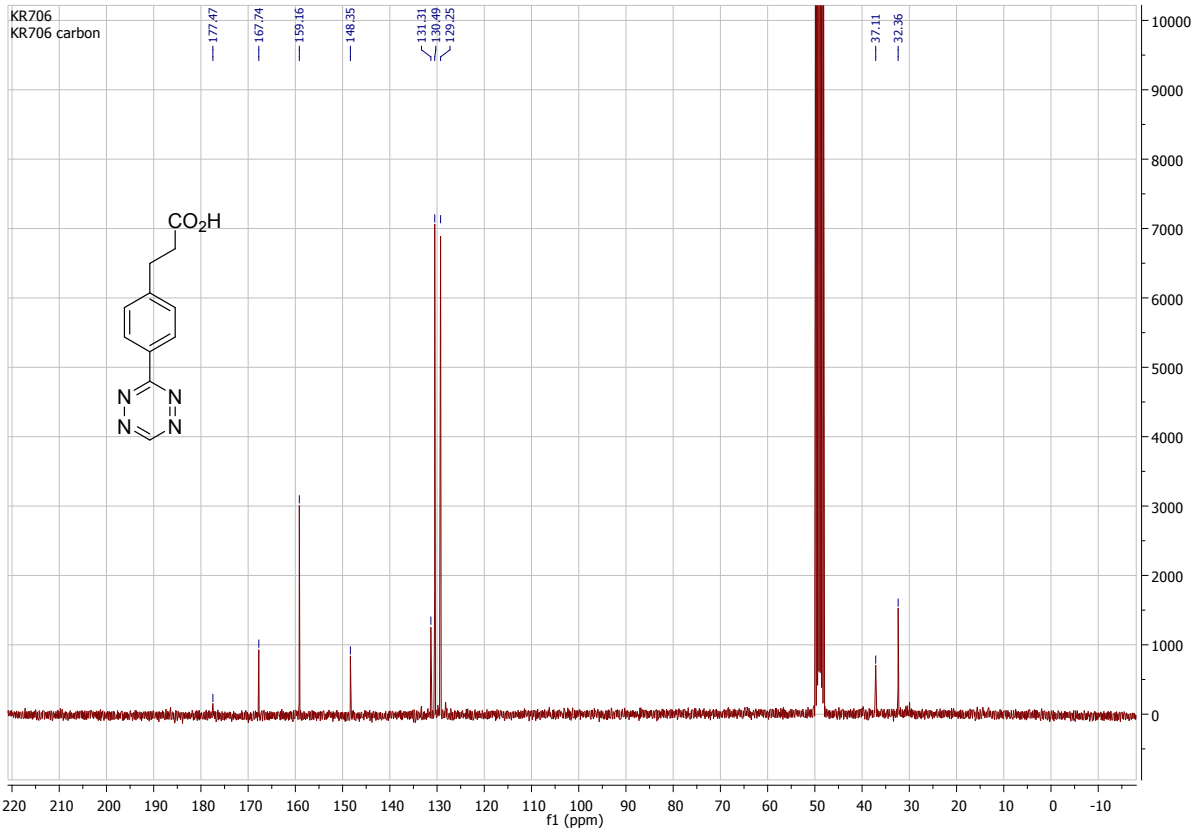
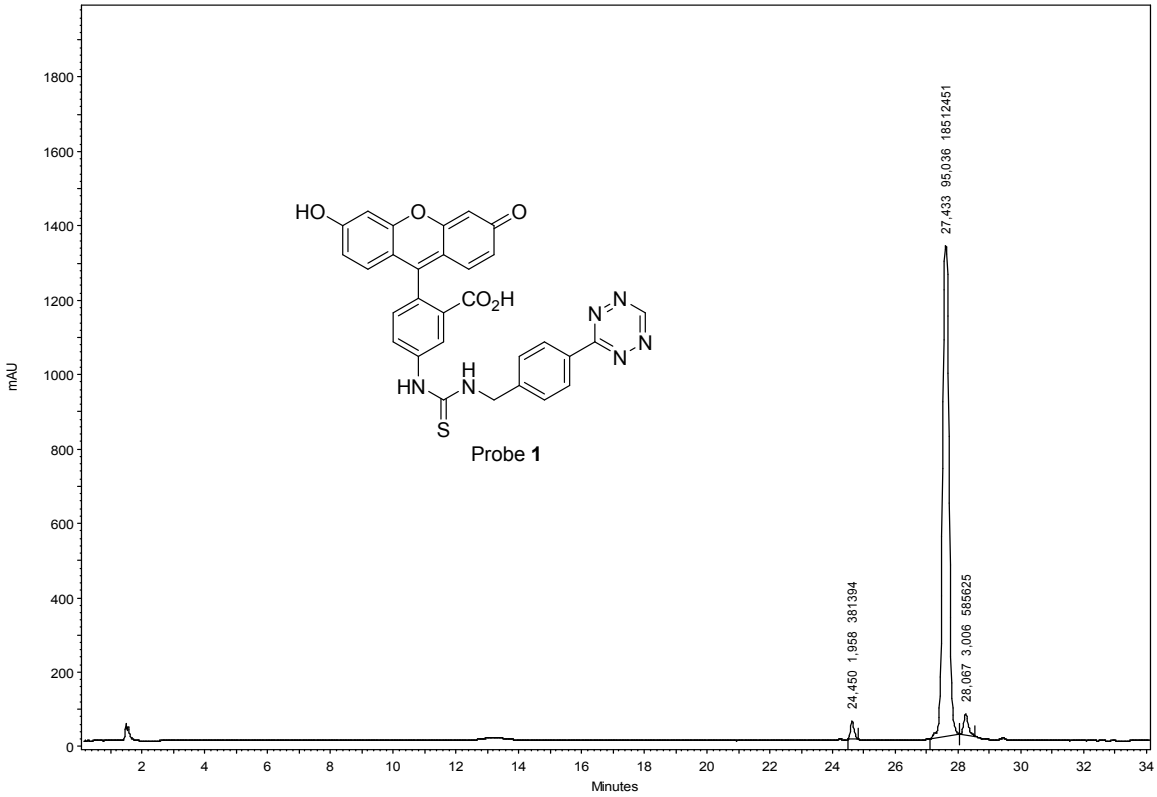
KR629 carbon
Pyr-Fluo 8
f1 (ppm)


Apr10-2018
KR706 proton
10.29
8.51
8.50
8.49
8.48
7.53
7.50
3.07
3.05
3.02
2.68
2.66
2.63
CO2H
N
N
N
N
1.00
1.96
2.03
2.07
2.05
f1 (ppm)

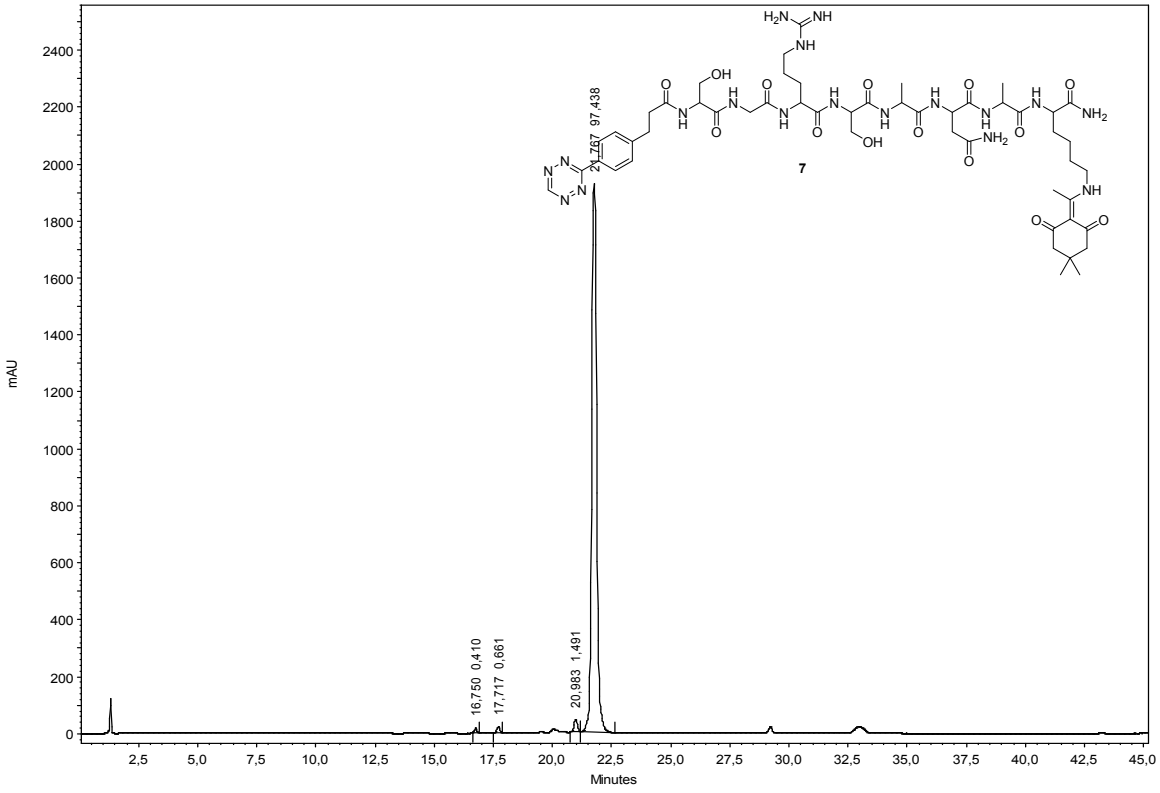

KR706
KR706 carbon
177.47
167.74
159.16
148.35
131.31
130.49
129.25
37.11
32.36
CO2H
N
N
N
N
f1 (ppm)

2.2) RP-HPLC elution profiles for compounds 1, 7-9

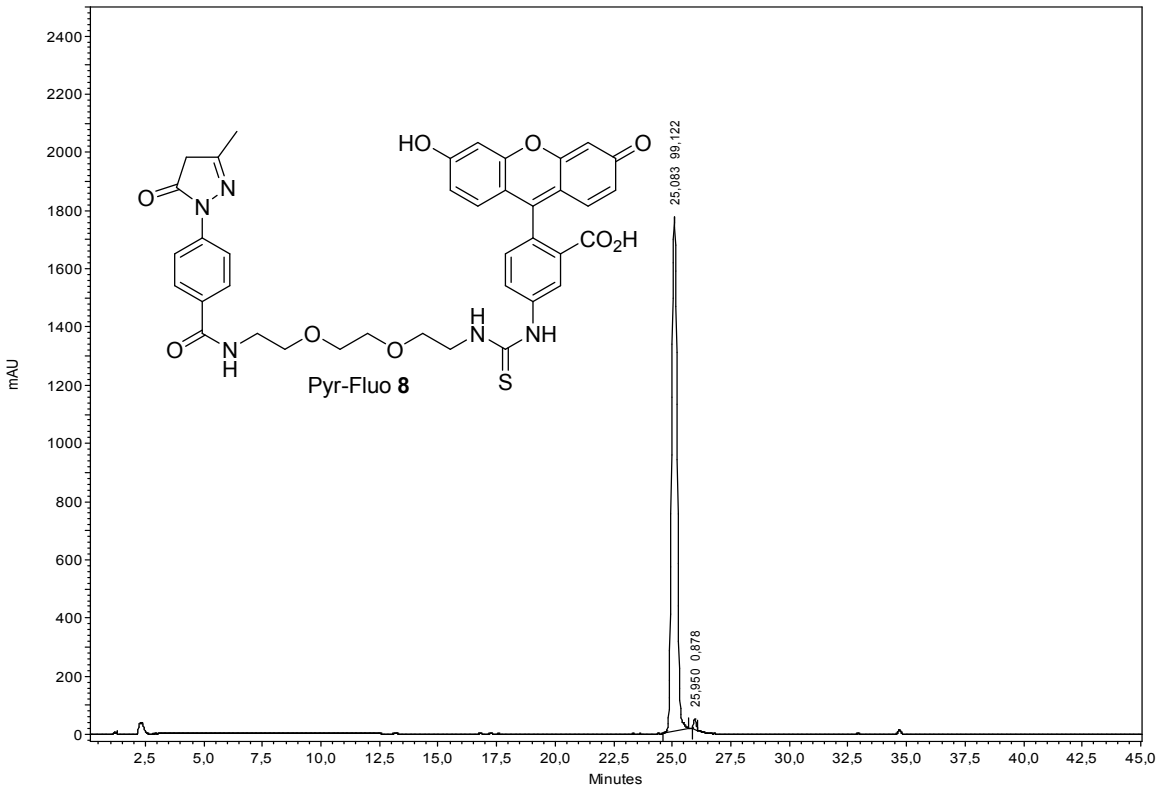
RP-HPLC elution profile of tetrazine-based probe **1** (recorded in "Max Plot" mode)



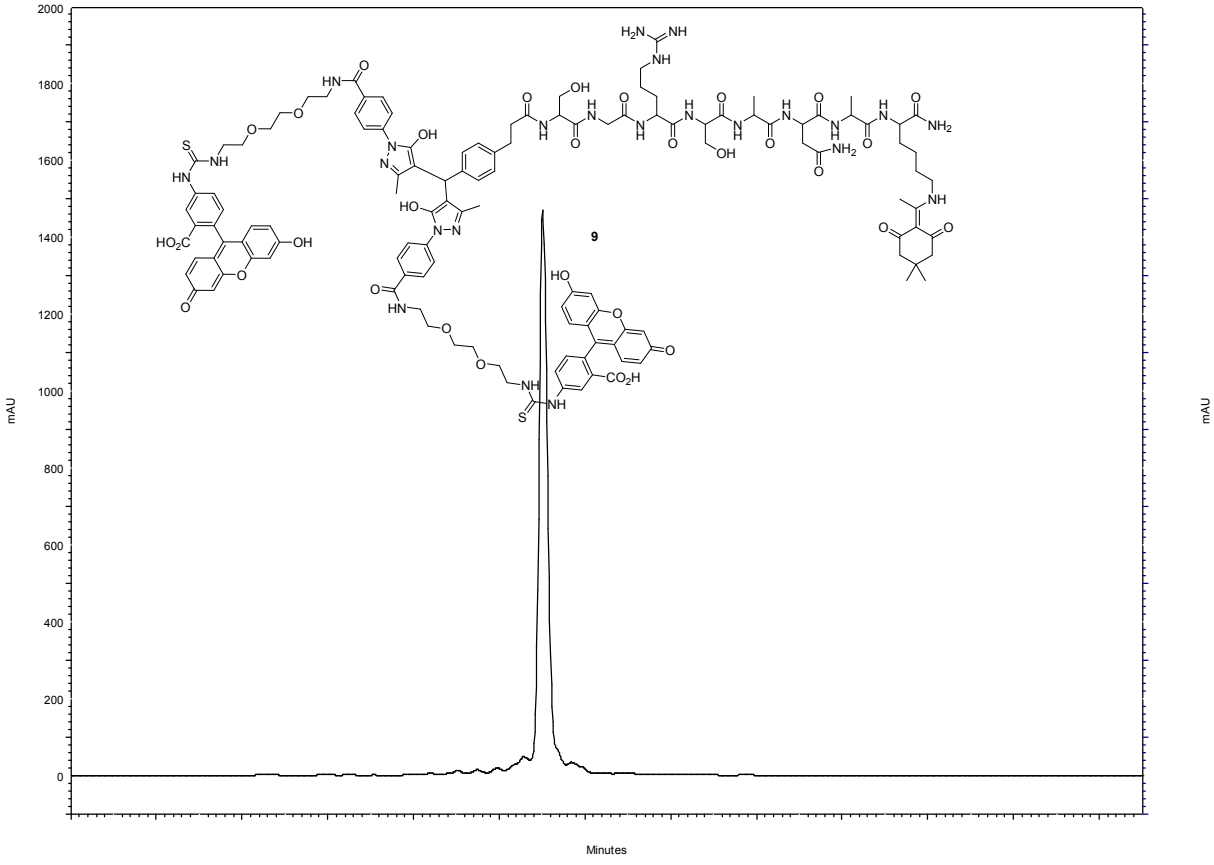
RP-HPLC elution profile of peptide **7** (recorded in "Max Plot" mode)



RP-HPLC elution profile of Pyr-Fluo **8** (recorded in "Max Plot" mode)



RP-HPLC elution profile of modified peptide **9** (recorded at 330 nm)



2.3) HRMS (ESI-TOF) for compound 9

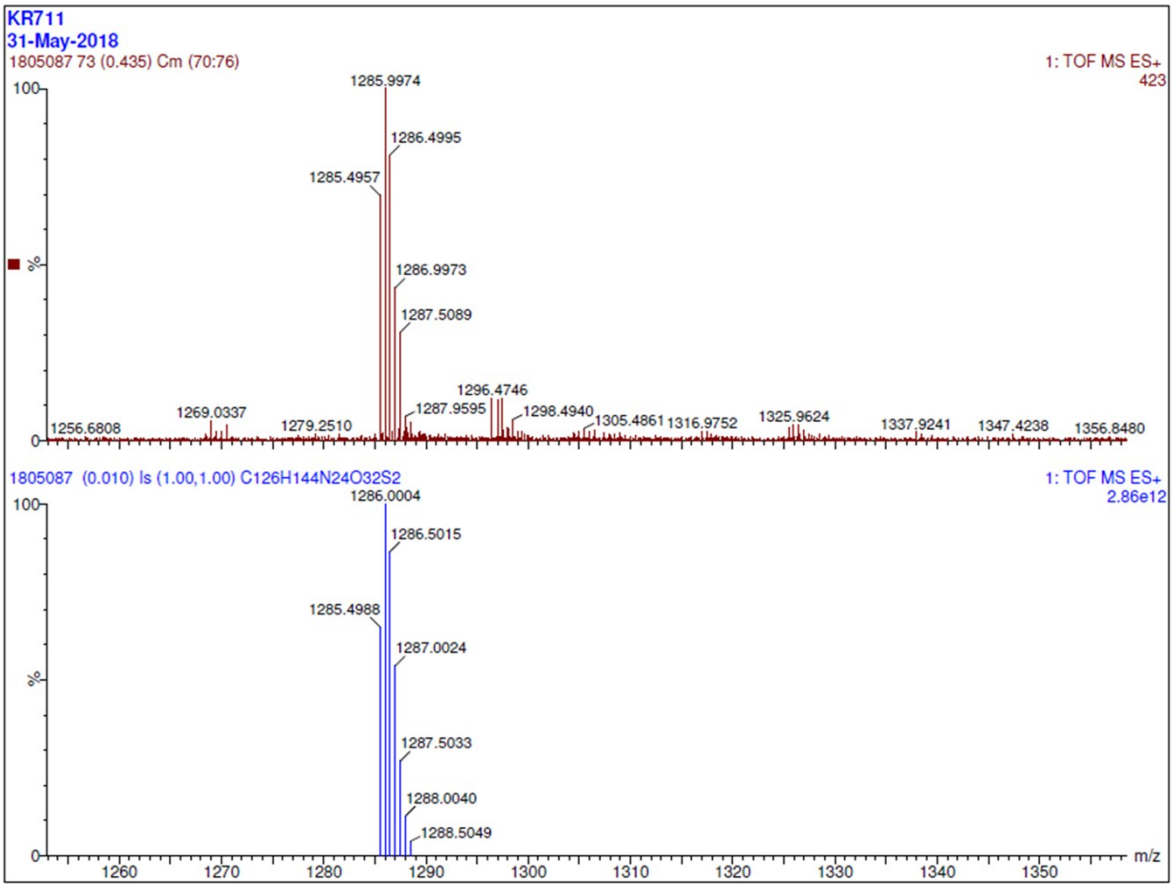
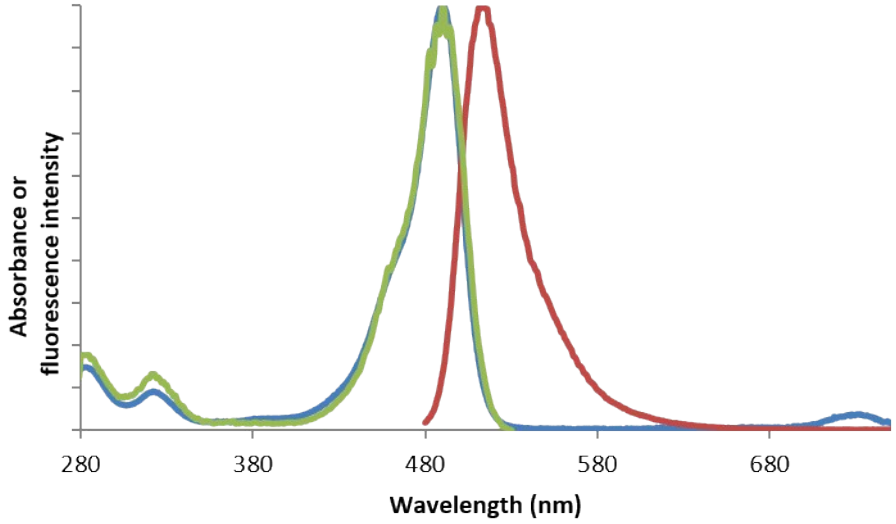
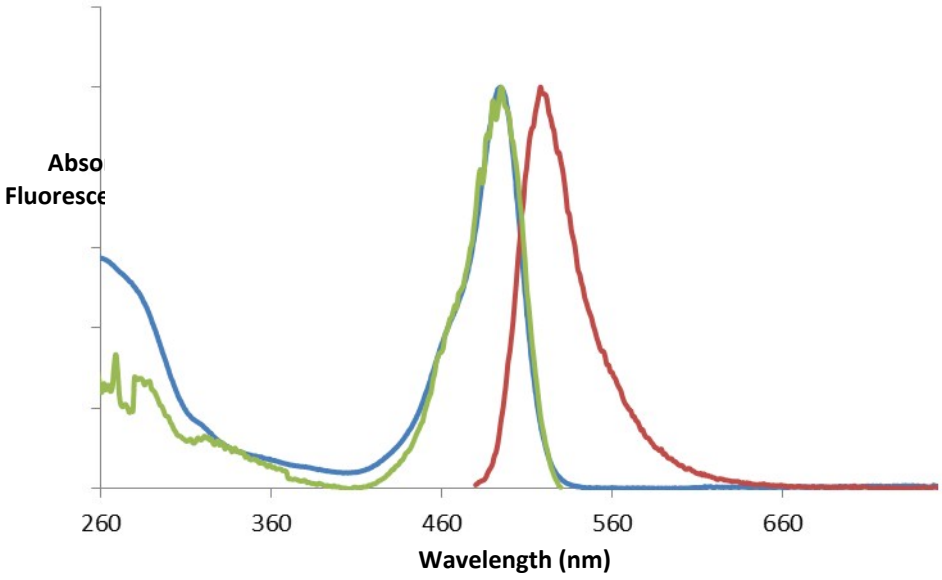


Figure S2: HRMS (ESI-TOF) exact mass calcd for $C_{126}H_{146}N_{24}O_{32}S_2$ m/z 1285.4988 (in blue); found m/z 1285.4957 (in red) $[M+2H]^{2+}$

2.4) Spectroscopic data for fluorescein, compounds 8, 9 and Lyso-Fluo

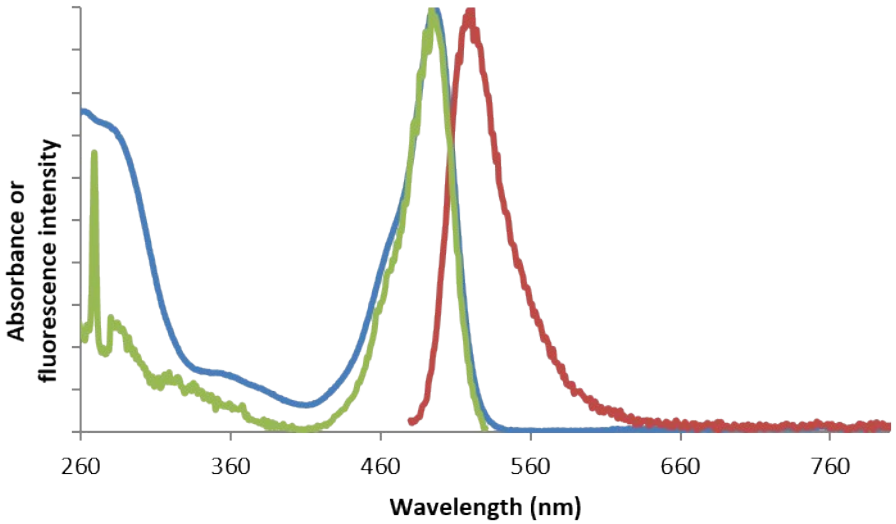


Normalized absorption (in blue), emission (in red, λ_{ex} 470 nm) and excitation (in green, λ_{em} 540 nm) spectra of fluorescein in PBS (0.1 M, pH 7.4) at 25 °C.

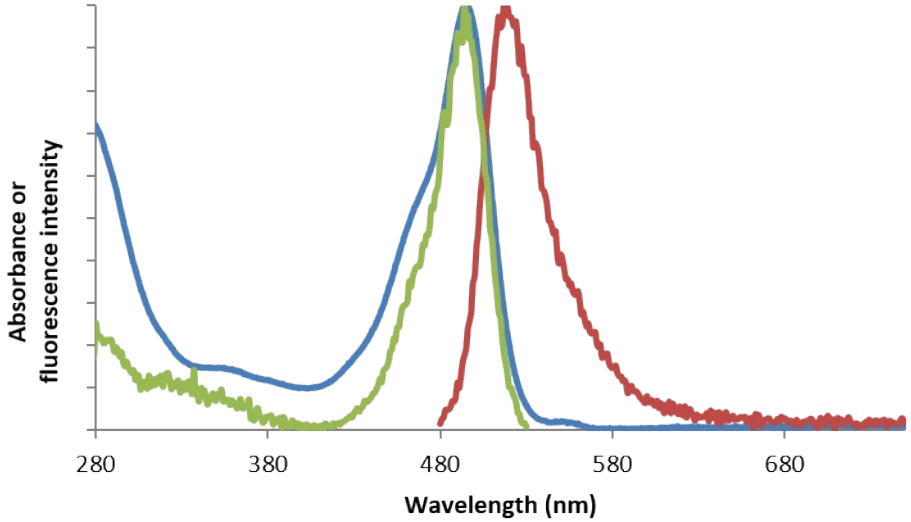


Normalized absorption (in blue), emission (in red, λ_{ex} 470 nm) and excitation (in green, λ_{em} 540 nm) spectra of

pyrazolone **8** in PBS (0.1 M, pH 7.4) at 25 °C.

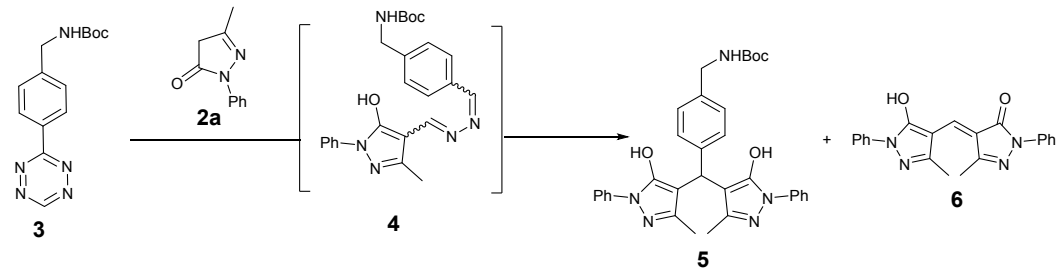


Normalized absorption (in blue), emission (in red, λ_{ex} 470 nm) and excitation (in green, λ_{em} 540 nm) spectra of peptide **9** in PBS (0.1 M, pH 7.4) at 25 °C.

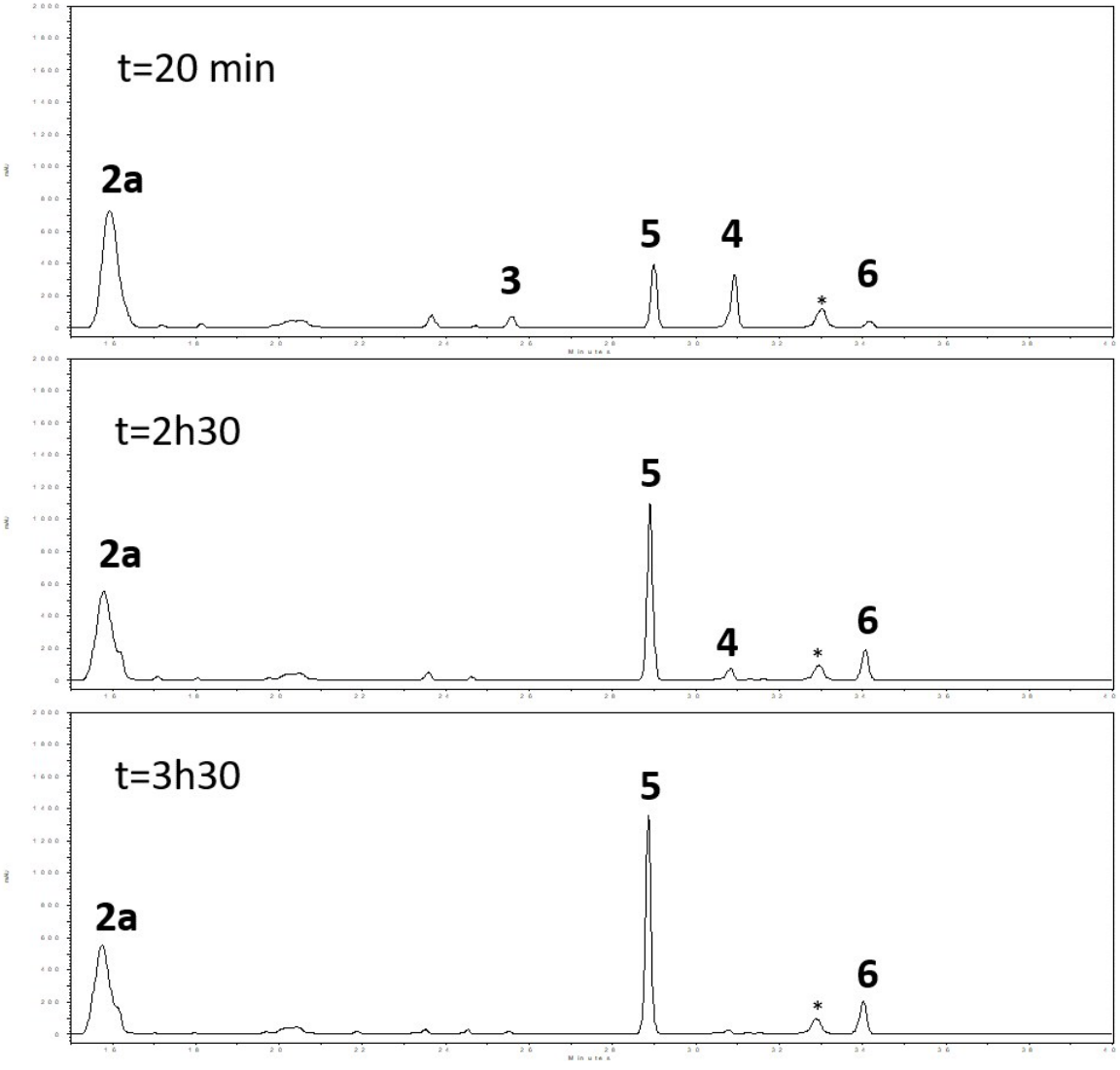


Normalized absorption (in blue), emission (in red, λ_{ex} 470 nm) and excitation (in green, λ_{em} 540 nm) spectra of Lyso-Fluo in PBS (0.1 M, pH 7.4) at 25 °C.

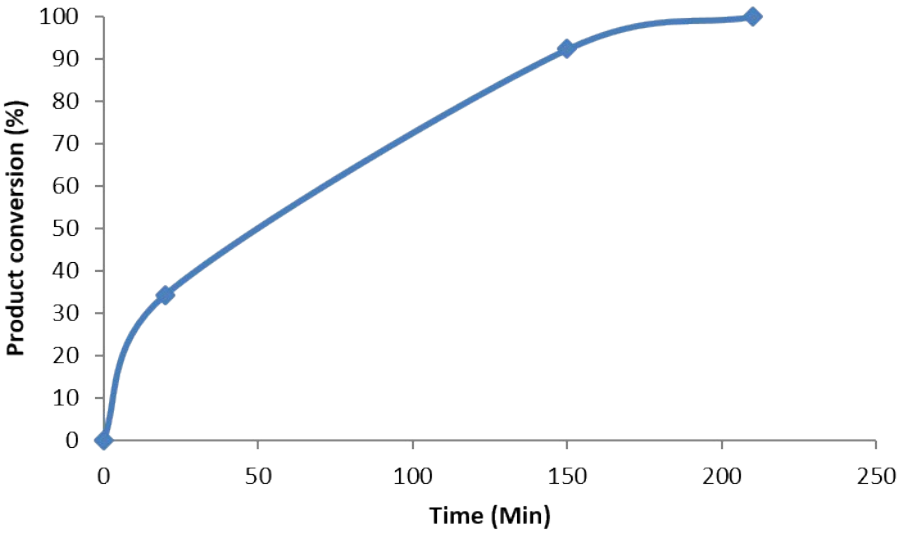
3) RP-HPLC monitoring of pyrazolone-tetrazine chemical ligation



To a solution of tetrazine **3** (25 mg, 0.09 mmol, 1 equiv.) in a 1.7 mL solution of ACN/PBS pH 7.4 (1:0.7, v/v) was added N-phenylpyrazolone **2a** (77 mg, 0.45 mmol, 5 equiv.). The reaction was stirred for 3.5 h at room temperature. A sample of the reaction mixture was collected for HPLC analysis at t=20 min, 2h30 and 3h30 (System QC, recorded in "Max Plot" mode, * contamination from TFA).



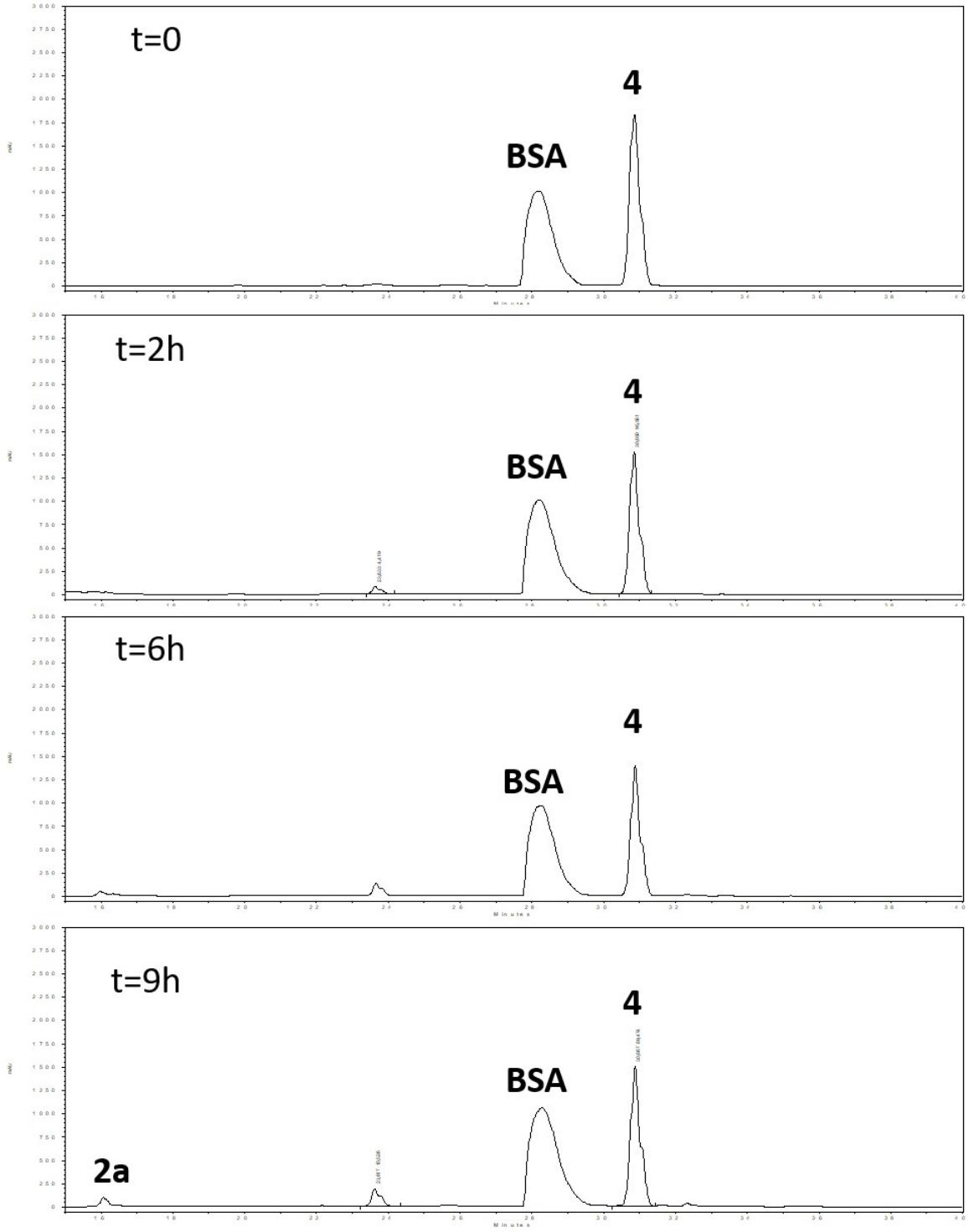
From these RP-HPLC chromatograms, the plot of conversion of product **6** as the function of reaction time was determined:



4) Tests of stability

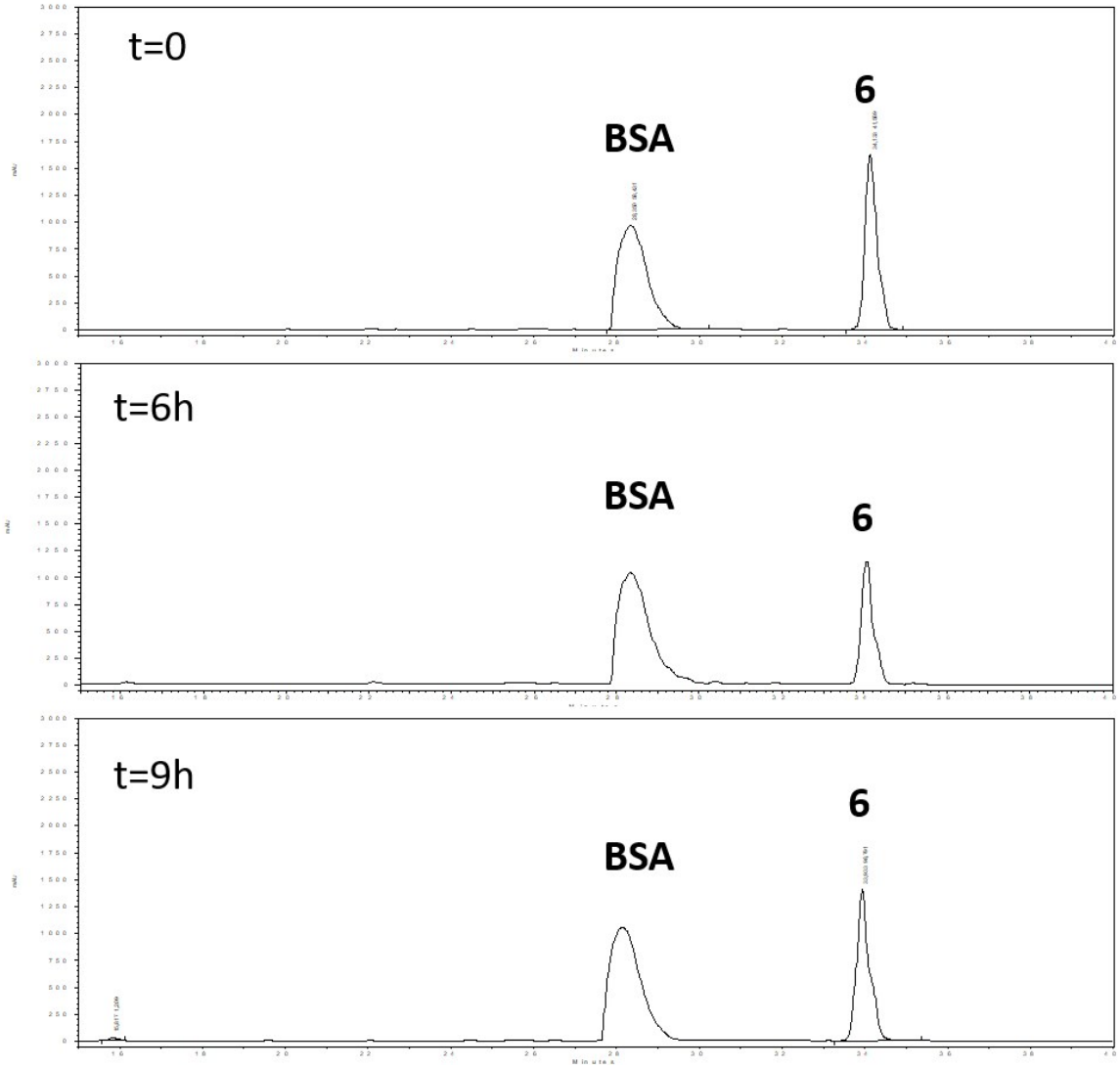
4.1) Study of stability of intermediate 4 in the presence of BSA

To a solution of bovine serum albumin (3.0 mg, 0.045 µmol) in PBS (0.1 M, pH 7.4, 900 µL) placed in an Eppendorf tube was added 100 µL of a stock solution of **4** (0.6 mg, 1.38 µmol, 500 µL acetonitrile). The resulting mixture was incubated at 37 °C. A sample of the reaction mixture was collected for HPLC analysis at t=0, 2, 6 and 9h (System QC, recorded in "Max Plot" mode).



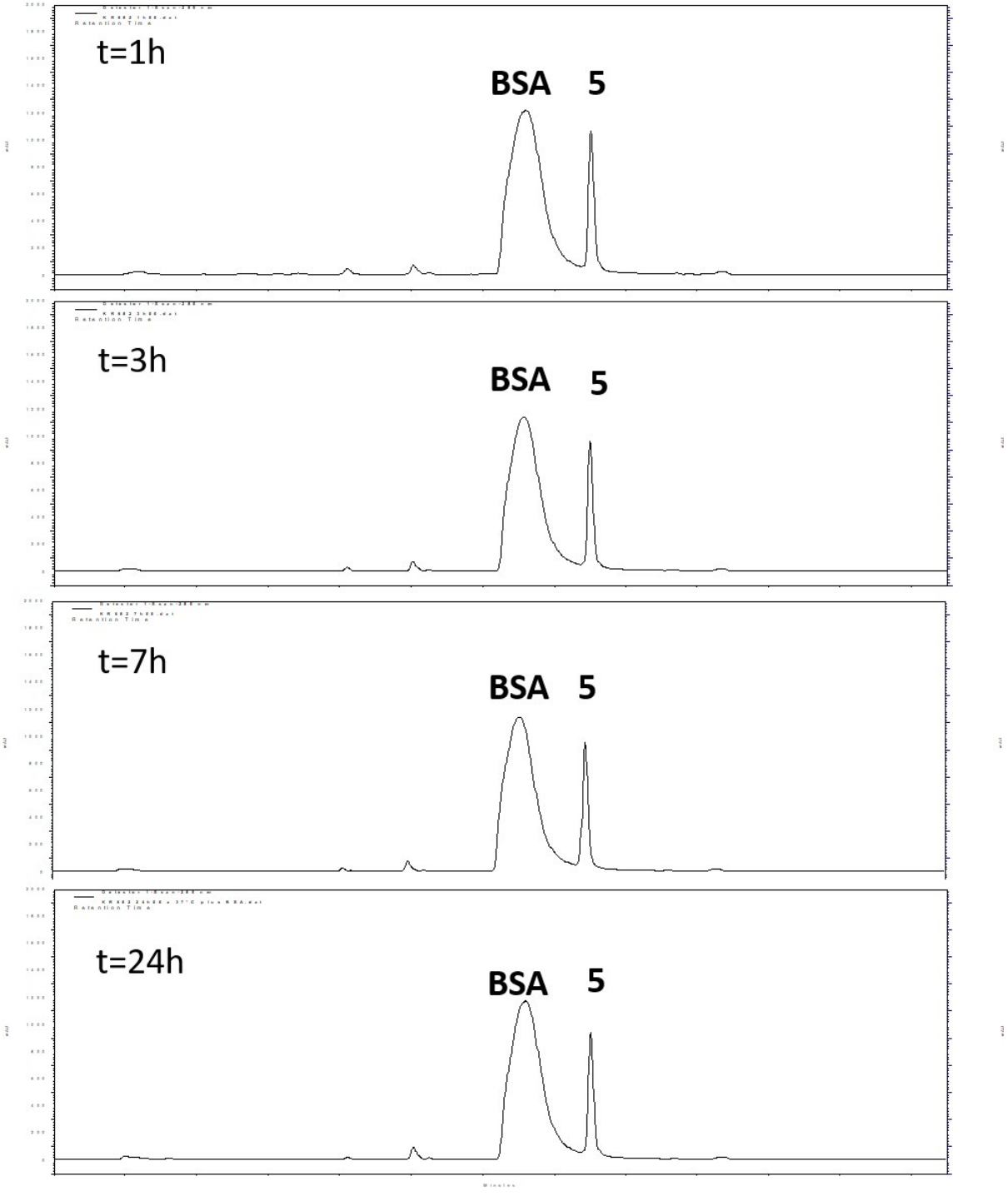
4.2) Study of stability of by-product 6 in the presence of BSA

To a solution of bovine serum albumin (3.0 mg, 0.045 µmol) in PBS (0.1 M, pH 7.4, 900 µL) placed in an Eppendorf tube was added 100 µL of a stock solution of **6** (0.5 mg, 1.36 µmol, 500 µL acetonitrile). The resulting mixture was incubated at 37 °C. A sample of the reaction mixture was collected for HPLC analysis at t=0, 6 and 9h (System QC, recorded in "Max Plot" mode).



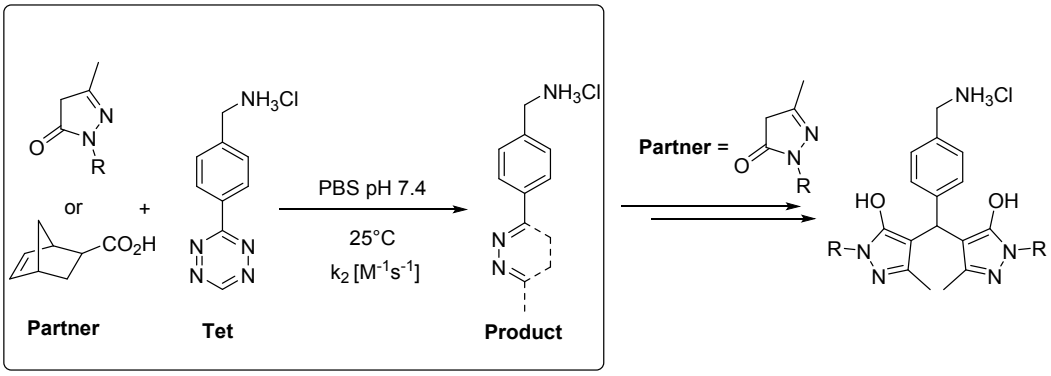
4.3) Study of stability of product 5 in the presence of BSA

To a solution of bovine serum albumin (14.5 mg, 0.220 µmol) in PBS (0.1 M, pH 7.4, 900 µL) placed in an Eppendorf tube was added 100 µL of a stock solution of **5** (0.625 mg, 1.10 µmol, 500 µL acetonitrile). The resulting mixture was incubated at 37 °C. A sample of the reaction mixture was collected for HPLC analysis at t=1, 3, 24h (System QC, recorded at 280 nm).



5) Determination of kinetic rate constants

5.1) Generalities



The reaction between tetrazine benzylamine tetrazine hydrochloride and the pyrazolones or 5-norbornene-2-carboxylic acid was followed by UV/Vis spectroscopy by measuring the absorbance decay of the tetrazine scaffold at 520 nm. Accordingly, only the kinetics of the first step of the reaction were determined.

Typical kinetic equation for a second order reaction is:

- $d[C]/dt = k_2.[\mathbf{Tet}].[\mathbf{Partner}]$

After integration, the following relation was found:

- $y = k_2 * t + \text{constant} = \{1/([\mathbf{Partner}]_0-[\mathbf{Tet}]_0)\} * \ln\{([\mathbf{Tet}]_0 * ([\mathbf{Partner}]_0 - [\text{Product}]_t)) / ([\mathbf{Partner}]_0 * ([\mathbf{Tet}]_0 - [\mathbf{Product}]_t))\}$

with :
- y in M^{-1}
- k_2 in $M^{-1}.s^{-1}$
- t in s
- [**Tet**], [**Partner**] and [**Product**] in M.

5.2) Experimental procedure

To a solution of benzylamine tetrazine hydrochloride (0.5 mM, 2.9 mL) in PBS pH 7.4 was added of a solution of the corresponding pyrazolone **(2a**, **2f**, or **2g)** or norbornene-2-carboxylic acid (19.5 mM, 100 µL) in DMSO. The resulting concentration of tetrazine and pyrazolone or norbornene in the 3mL solution of PBS/DMSO (29:1, v/v) was 0.48 mM and 0.65 mM, respectively. The kinetics were monitored at 25 °C by following the decrease in absorbance of tetrazine at 520 nm every 0.5 s.

5.3) Measurement of the kinetic rate constants of disappearance of the tetrazine

Phenyl pyrazolone 2a

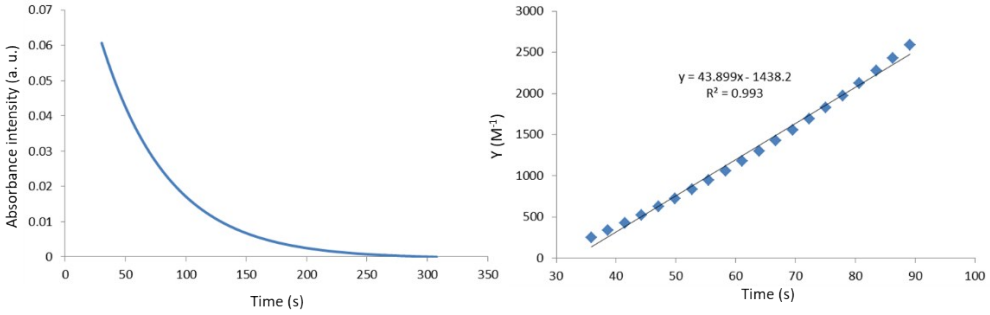
The absorbance value at the plateau was correlated to the final concentration of product as follows:

$$\text{Final absorbance intensity} = [\textbf{Product}]_{final} = [\textbf{Tet}]_0 = 0.5 \text{ mM}$$

This correlation was employed so as to calculate the product concentration [**Product**] at any time.

The y values at any time were calculated with the following equation (only true for the linear part of the curve [**Product**] = f(t), from 30 s to 90 s):

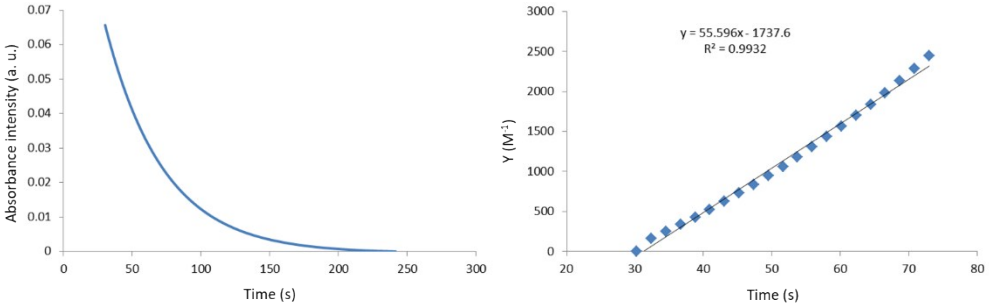
- $[\textbf{Tet}]_0 = 0.5$ mM
- $[\textbf{Partner}]_0 = 0.65$ mM
- $[\textbf{Product}]_t$ = values from the linear part of [**Product**] against Time (range from 30 s to 90 s).



The linear regression affords a slope = $k_2 = 43 \pm 1$ M^{-1} s^{-1}.

Sulfopyrazolone 2g

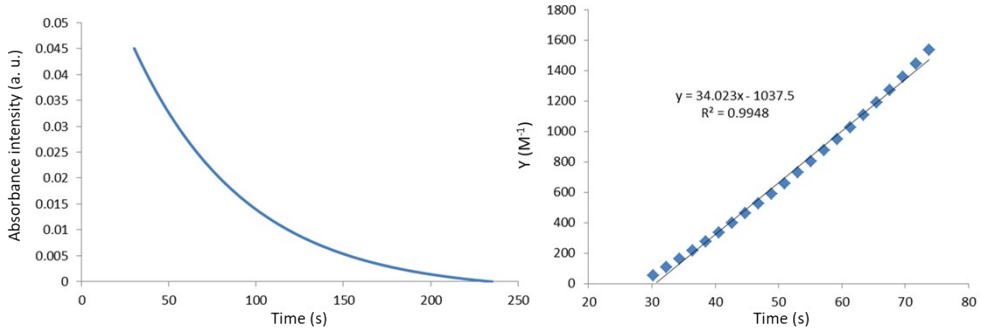
- $[\textbf{Tet}]_0 = 0.5$ mM
- $[\textbf{Partner}]_0 = 0.65$ mM
- $[\textbf{Product}]_t$ = values from the linear part of [**Product**] against Time (range from 30 to 75 s).



The linear regression affords a slope = $k_2 = 55 \pm 1$ M^{-1} s^{-1}.

Methyl pyrazolone 2f

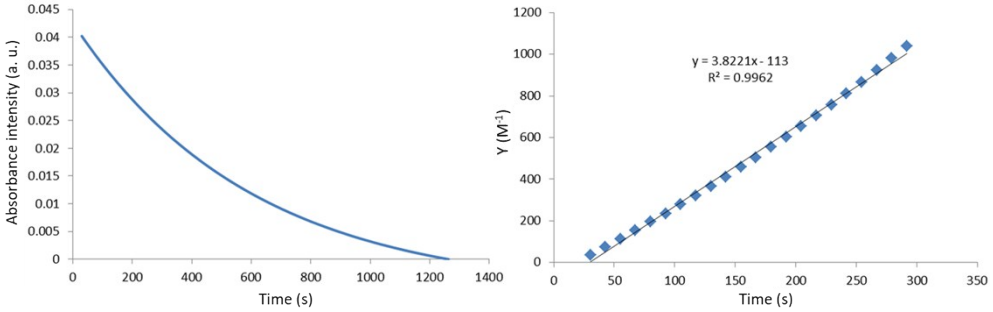
- $[\textbf{Tet}]_0 = 0.5$ mM
- $[\textbf{Partner}]_0 = 0.65$ M
- $[\textbf{Product}]_t$ = values from the linear part of [**Product**] against Time (range from 30 to 75 s).



The linear regression affords a slope = k_2 = **34 ± 1 M^{-1} s^{-1}**.

<u>Norbornene-2-carboxylic acid</u>

- [**Tet**]$_0$ = 0.5 mM
- [**Partner**]$_0$ = 0.65 M
- [**Product**]$_t$ = values from the linear part of [**Product**] against Time (range from 30 to 304 s).



The linear regression affords a slope = k_2 = **3.8 ± 0.1 M^{-1}.s^{-1}**.

<u>Table S1.</u> Second order rate constant of benzylamine tetrazine hydrochloride with pyrazolones or 5-norbornene-2-carboxylic acid in PBS pH 7.4, 25 °C

Partner	R^1	k_2 [M^{-1}.s^{-1}]
2a	Ph	43 ± 1
2f	Me	34 ± 1
2g	Ph-*p*SO_3H	55 ± 1
5-Norbornene-2-carboxylic acid		3.8 ± 0.1

6) Determination of D/P ratio of labelled human lysozyme

The degree of labelling of human lysozyme (Lyso-Fluo), i.e. the Dye/Protein (D/P) molar ratio, was estimated from the relative intensities of protein (h-Lyso) and dye absorption (Pyr-Fluo), according to the following equation:

$$\frac{D}{P} = \frac{c_D}{c_P} = \frac{A_D/\varepsilon_D}{(A_{280} - \varepsilon_{280}^{'} \cdot c_D)/\varepsilon_{280}}$$

Where C_D and C_P is the concentration of the dye and protein moiety respectively, in the Lyso-Fluo conjugate. C_D is obtained from the absorbance at the dye's long wavelength absorbance A_D (494 nm). ε_D (52518 M^{-1} cm^{-1}), $\varepsilon_{280}^{'}$ (25953 M^{-1} cm^{-1}) are the molar extinction coefficients of the dye at 494 nm and 280 nm, respectively. ε_{280} (23441 M^{-1} cm^{-1}) is the molar extinction coefficient of h-Lyso at 280 nm (39914 $L.mol^{-1}.cm^{-1}$). The following Table summarizes the data for the calculation.

	Lyso-FLuo
$\varepsilon_{280}^{'}$ **(L.mol^{-1}.cm^{-1})**	25953
ε_D **(L.mol^{-1}.cm^{-1})**	52518
ε_{280} **(L.mol^{-1}.cm^{-1})**	23441
A_{280}	0.093
A_D	0.109
C_D **(mol/L)**	$2.08 \cdot 10^{-6}$
C_P **(mol/L)**	$1.69 \cdot 10^{-6}$
D/P	1.24