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Supporting information

Structure-reactivity Relationship Study of Cyclo-dioxo Maleimide Cross-linker for Serum Stability and Self-hydrolysis.

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Contents

Chemical synthesis

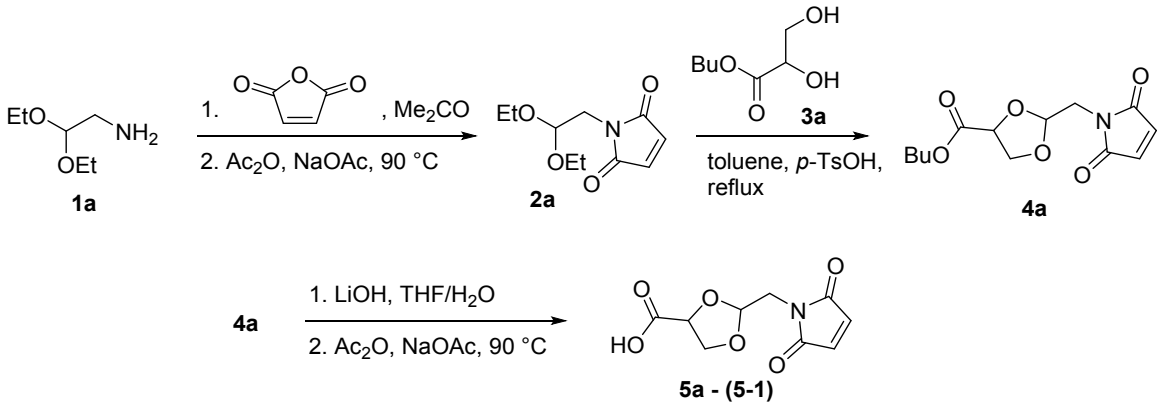
General experimental procedures

Unless otherwise indicated, reactions were carried out under an atmosphere of argon in flame-dried glassware with magnetic stirring. Air and/or moisture-sensitive liquids were transferred via syringe. When required, solutions were degassed by bubbling of argon through a needle. Organic solutions were concentrated by rotary evaporation at 25-60 °C at 15-30 torr. Analytical thin layer chromatography (TLC) was performed using plates cut from glass sheets (silica gel 60F-254 from Merck). Visualization was achieved under a 254 or 365 nm UV light and by immersion in an appropriate revelation solution. Column chromatography was carried out as "Flash Chromatography" using silica gel G-25 (40-63 µm) from Macherey-Nagel.

Materials and methods

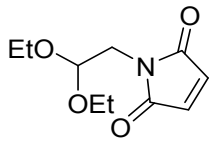
All reagents were obtained from commercial sources and used without any further purifications. Anhydrous solvents used in experiments were obtained from Sigma-Aldrich or Alfa Aesar. ^{1}H and ^{13}C NMR spectra were recorded respectively at 400 MHz and 100 MHz with a Bruker 400 spectrometer at 23 °C. Chemical shifts are reported in parts per million (δ) and calibrated using residual non-deuterated solvent. Data are represented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, m = multiplet, br = broad or a combination of the above), coupling constant (J, Hz) and integration. High resolution mass spectra were obtained using an Agilent Q-TOF (time of flight) 6520. Low resolution mass spectra were obtained using an Agilent MSD 1200 SL (ESI/APCI) with a Agilent HPLC1200 SL and a Waters Acquity QDa (ESI) with a Waters Alliance 2695 HPLC.
Preparative HPLC procedures were performed on semi-preparative HPLC Shimadzu Auto-injector SIL-10A (pump: Shimadzu LC-8A, UV-Vis detector: Shimadzu SPD-10A, collector: Shimadzu fraction collector FRC-10A) using a Sunfire C18 (150 mm × 19 mm i.d., 5 µm, Waters) at a flow of 17 mL/min. 1 mL of sample was injected and water/ACN (containing 0.05% TFA) was used as eluent system. The gradient applied was 5% to 95% ACN in 40 minutes and 10 minutes of re-equilibration. Detection was done at 550 nm for TAMRA derivatives.

Synthesis of linker (5-1)



Scheme S1: synthesis of linker **5-1**

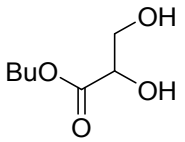
1-(2,2-diethoxyethyl)-1H-pyrrole-2,5-dione, 2a



Chemical Formula: $C_{10}H_{15}NO_4$
Molecular Weight: 213,23

Molecule **2a** was synthesized according to the reported procedures.[1]

butyl 2,3-dihydroxypropanoate, 3a

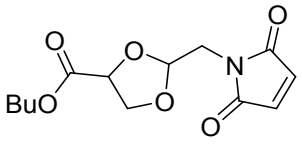


Chemical Formula: $C_7H_{14}O_4$
Molecular Weight: 162,19

A solution of picolinic acid (0.018 eq., 18 mM, 15.6 mL) in acetone and a solution of manganese (II) acetate (0.003 eq., 3 mM, 15.6 mL) in acetone were added subsequently to a solution of butyl acrylate (1 eq., 2 g, 15.6 mmol) in acetone (50 mL) at r.t. Then a solution of sodium acetate (0.03 eq., 0.6 M, 0.78 mL) in water was added to the reaction mixture and the temperature was lowered to 0 °C. 3.54 mL of 30% aqueous solution of H_2O_2 (2 eq., 31.2 mmol) was added using a syringe pump at a rate of 0.5 mL/h. The resulting mixture was stirred for 16 h allowing the temperature to raise to r.t. After the reaction was complete, the mixture was poured in saturated aqueous solution of $NaHCO_3$, the aqueous layer was extracted with DCM, the combined organic layers were dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The residue was filtrated on a silica pad using cyclohexane to remove the remaining starting material and then using ethyl acetate to obtain **3a** as a dense transparent-white liquid in 71% yield.

^{1}H NMR (400MHz, $CDCl_3$, δ ppm): 4.25 (t, J = 3.4 Hz, 1H), 4.20 (t, J = 6.7 Hz, 2H), 3.85 (ddd, J = 15.6, 11.7, 3.5 Hz, 2H), 3.58 (br. s., 1H), 2.78 (br. s., 1H), 1.70 – 1.56 (m, 2H), 1.45 – 1.31 (m, 2H), 0.92 (t, J = 7.4 Hz, 3H).
^{13}C NMR (100MHz, $CDCl_3$, δ ppm): 173.13, 71.72, 65.93, 64.14, 30.53, 19.00, 13.62.
HR-ESI-MS $C_7H_{14}O_4$ 162.08921 found 162.08853

butyl 2-((2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)methyl)-1,3-dioxolane-4-carboxylate, 4a



Chemical Formula: $C_{13}H_{17}NO_6$
Molecular Weight: 283,28

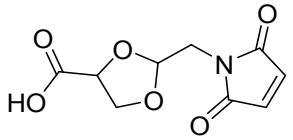
A solution of **2a** (1 eq., 677 mg, 3.18 mmol) and **3a** (1 eq., 515 mg, 3.18 mmol) in toluene (50 mL) containing a catalytic amount of *p*-toluensulfonic acid monohydrate (0.2 eq., 120 mg, 0.635 mmol) was refluxed for 2 h. Ethanol was removed as azeotrope of toluene (b.p. of azeotrope: 76.7°C) and the reaction was monitored by TLC. After disappearing of the starting material, toluene was evaporated at

reduced pressure, the residue was redissolved in ethyl acetate and washed with a saturated solution of $NaHCO_3$ and brine. The organic phase was dried over Na_2SO_4 and the solvent was evaporated. The crude product was purified by flash chromatography (cyclohexane, then cyclohexane to EtOAc), yielding the wanted product **4a** in 54% yield and **ethyl 2-((2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)methyl)-1,3-dioxolane-4-carboxylate** as by-product (21%), which will be used as well for the following step.

butyl 2-((2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)methyl)-1,3-dioxolane-4-carboxylate, mixture of *cis*- and *trans*- isomers.
^{1}H NMR (400MHz, $CDCl_3$, δ ppm): 6.73 (s, 4H, *cis* + *trans*), 5.35 (t, J = 4.5 Hz, 1H, *trans*), 5.23 (t, J = 3.6 Hz, 1H, *cis*), 4.66 – 4.59 (m, 1H, *trans*), 4.54 (dd, J = 7.2, 4.0 Hz, 1H, *cis*), 4.27 (t, J = 7.9 Hz, 1H, *trans*), 4.22 – 4.19 (m, 1H, *cis*), 4.15 (q, J = 13.4, 6.7 Hz, 4H, *cis* + *trans*), 4.08 (t, J = 8.1 Hz, 1H, *cis*), 3.95 (dd, J = 8.3, 5.3 Hz, 1H, *trans*), 3.87 (d, J = 4.2 Hz, 2H, *cis*), 3.74 (d, J = 4.4 Hz, 2H, *trans*), 1.68 – 1.58 (m, 4H, *cis* + *trans*), 1.37 (dp, J = 14.3, 7.2 Hz, 4H, *cis* + *trans*), 0.93 (td, J = 7.2, 3.5 Hz, 6H, *cis* + *trans*).
^{13}C NMR (100MHz, $CDCl_3$, δ ppm): 170.54, 170.34, 170.19, 134.26, 134.22, 102.68, 101.96, 74.05, 73.96, 68.64, 68.00, 65.44, 39.70, 39.54, 30.53, 19.02, 13.66, 13.63.
HR-ESI-MS $C_{13}H_{17}NO_6$ 283.10559 found 283.10564

ethyl 2-((2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)methyl)-1,3-dioxolane-4-carboxylate, mixture of *cis*- and *trans*- isomers.
^{1}H NMR (400MHz, $CDCl_3$, δ ppm): 6.74 (s, 4H, *cis* + *trans*), 5.36 (t, J = 4.6 Hz, 1H, *trans*), 5.25 – 5.21 (m, 1H, *cis*), 4.62 (dd, J = 7.2, 5.3 Hz, 1H, *trans*), 4.54 (dd, J = 7.5, 3.9 Hz, 1H, *cis*), 4.27 (dd, J = 8.4, 7.4 Hz, 1H, *trans*), 4.20 (ddt, J = 7.2, 4.6, 2.4 Hz, 4H, *cis* + *trans*), 4.16 (dd, J = 8.2, 4.2 Hz, 1H, *cis*), 4.07 (dd, J = 8.8, 7.5 Hz, 1H, *cis*), 3.95 (dd, J = 8.5, 5.3 Hz, 1H, *trans*), 3.87 (dd, J = 3.7, 3.0 Hz, 2H, *cis*), 3.74 (d, J = 4.6 Hz, 2H, *trans*), 1.28 (td, J = 7.1, 2.8 Hz, 6H, *cis* + *trans*).
^{13}C NMR (100MHz, $CDCl_3$, δ ppm): 170.45, 170.35, 170.27, 170.20, 134.27, 134.23, 102.67, 101.97, 74.02, 73.95, 68.64, 67.97, 61.61, 61.58, 39.68, 39.52, 14.14, 14.13**.**
HR-ESI-MS $C_{11}H_{13}NO_6$ 255.07429 found 255.07428

2-((2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)methyl)-1,3-dioxolane-4-carboxylic acid, 5a – (5-1)



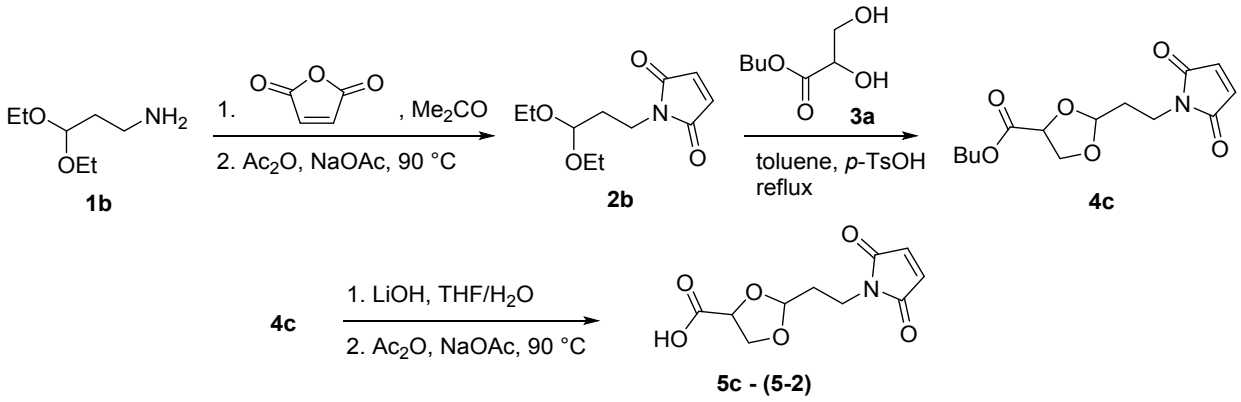
Chemical Formula: $C_9H_9NO_6$
Molecular Weight: 227,17

A solution of LiOH (14 eq., 219 mg, 9.16 mmol) in water (7 mL) was poured to a solution of ethyl 2-[(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)methyl]-1,3-dioxolane-4-carboxylate (1 eq., 167 mg, 0.654 mmol) and **4a** (2.61 eq., 483 mg, 1.71 mmol) in THF (10 mL) and the reaction mixture was stirred for 30 minutes at r.t.. Completion of the reaction was checked by TLC, then EtOAc was added and the mixture was acidified with aqueous 3M solution of HCl to pH 2. The aqueous phase was extracted with EtOAc and the combined organic layers were washed with water and with brine, dried over Na_2SO_4 and concentrated to give the intermediate product used in the next step without further purification.
The intermediate product 2-{[(2Z)-3-carboxyprop-2-enamido]methyl}-1,3-dioxolane-4-carboxylic acid (1 eq., 165 mg, 0.673 mmol) was treated with sodium acetate (2.4 eq., 132 mg, 1.62 mmol) in acetic anhydride (10 mL). The mixture was stirred for 15 min at r.t. and then for 2 h at 80 °C. The acetic anhydride was evaporated under reduced pressure and 5 mL of water were added. The mixture was stirred for 30 min at r.t. and then extracted with ethyl acetate. The solvent was evaporated and the

resulting crude material was purified by preparative HPLC to afford **5a** as a light-yellow oil in 37% overall yield (mixture of *cis*- and *trans*-isomers).

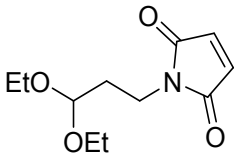
^{1}H NMR (400MHz, MeOH-d4, δ ppm): 6.87 (s, 2H, *trans*), 6.85 (s, 2H, *cis*), 5.29 (s, 1H, *trans*), 5.20 (s, 1H, *cis*), 4.66 (t, J = 5.4 Hz, 1H, *trans*), 4.57 (bs, 1H, *cis*), 4.29 (t, J = 7.8 Hz, 1H, *trans*), 4.16 (bs, 1H, *cis*), 4.11 (t, J = 8.2 Hz, 1H, *cis*), 4.01 – 3.94 (m, 1H, *trans*), 3.78 (t, J = 12.7 Hz, 2H, *cis*), 3.70 (d, J = 2.7 Hz, 2H, *trans*).
^{13}C NMR (100MHz, MeOH-d4, δ ppm): 170.77, 170.63, 134.13, 102.47, 101.70, 73.66, 68.23, 67.59, 39.27, 39.14.
HR-ESI-MS $C_9H_9NO_6$ 227.04299 found 227.04251

Synthesis of linker (5-2)



Scheme S2: synthesis of linker **5-2**

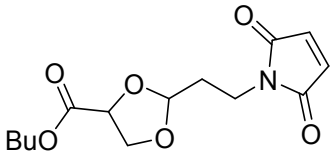
1-(3,3-diethoxypropyl)-1H-pyrrole-2,5-dione, 2b



Chemical Formula: $C_{11}H_{17}NO_4$
Molecular Weight: 227,26

Maleic anhydride (1 eq., 3.31 g, 33.7 mmol) was dissolved in acetone (23.2 mL) and 1-amino-3,3-diethoxypropane (1 eq., 4.97 g, 5.46 mL, 33.7 mmol) was added at 0 °C. The mixture was stirred for five minutes, then the solvent was evaporated to afford a crude residue. The residue was dissolved in acetic anhydride (6.8 mL) and sodium acetate (1.2 eq., 200 mg, 2.45 mmol) was added. The reaction mixture was warmed up to 90 °C and stirred for 2 h. (N.B. prolonging the reaction time leads to product decomposition). The reaction mixture was then filtrated with toluene and the solvent was evaporated. The obtained dark brown liquid was purified by flash chromatography (cyclohexane, then cyclohexane to EtOAc), to give product **2b** as a yellow liquid in overall yield of 47%.
^{1}H NMR (400MHz, $CDCl_3$, δ ppm): 6.68 (s, 2H), 4.50 (t, J = 5.4 Hz, 1H), 3.60 (q, J = 6.7 Hz, 4H), 3.52 – 3.40 (m, 2H), 1.90 (q, J = 6.4 Hz, 2H), 1.17 (t, J = 7.0 Hz, 6H).
^{13}C NMR (100MHz, $CDCl_3$, δ ppm): 169.69, 133.13, 100.03, 60.25, 33.10, 31.34, 14.30.
HR-ESI-MS $C_{11}H_{17}NO_4$ 227.11576 found 227.11531

butyl 2-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)-1,3-dioxolane-4-carboxylate, 4c



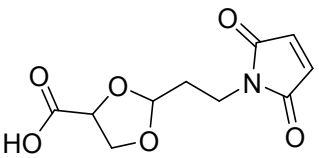
Chemical Formula: $C_{14}H_{19}NO_6$
Molecular Weight: 297,31

A solution of **2b** (1 eq., 271 mg, 1.19 mmol) and **3a** (1 eq., 193 mg, 1.19 mmol) in toluene (3.97 mL) containing a catalytic amount of *p*-toluensulfonic acid monohydrate (0.2 eq., 45.4 mg, 0.238 mmol) was refluxed for 2 h. Ethanol was removed as azeotrope of toluene (b.p. of azeotrope: 76.7°C) and the reaction was monitored by TLC. After disappearing of the starting material, toluene was evaporated at reduced pressure. The residue was dissolved in ethyl acetate and washed with a saturated solution of $NaHCO_3$ and brine. The organic phase was dried over Na_2SO_4 and the solvent was evaporated. The crude product was purified by flash chromatography (cyclohexane, then cyclohexane to EtOAc) to afford **4c** in 51% yield and **ethyl 2-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)-1,3-dioxolane-4-carboxylate** as by-product (45%), which will be used as well for the following step.

butyl 2-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)-1,3-dioxolane-4-carboxylate, mixture of *cis*- and *trans*-isomers.
^{1}H NMR (400MHz, $CDCl_3$, δ ppm): 6.67 (s, 4H, *cis* + *trans*), 5.12 (t, J = 3.7 Hz, 1H, *trans*), 5.05 (t, J = 4.5 Hz, 1H, *cis*), 4.56 (t, J = 6.9 Hz, 1H, *trans*), 4.51 (dd, J = 7.6, 3.5 Hz, 1H, *cis*), 4.28 (t, J = 8.0 Hz, 1H, *trans*), 4.16 (t, J = 6.6 Hz, 4H, *cis* + *trans* + 1H, *cis*), 4.02 (t, J = 8.1 Hz, 1H, *cis*), 3.87 – 3.79 (m, 1H, *trans*), 3.77 – 3.66 (m, J = 19.1, 6.7 Hz, 4H, *cis* + *trans*), 2.15 – 1.99 (m, 4H, *cis* + *trans*), 1.69 – 1.59 (m, 4H, *cis* + *trans*), 1.38 (dt, J = 14.9, 7.6 Hz, 4H, *cis* + *trans*), 0.93 (t, J = 7.3 Hz, 6H, *cis* + *trans*).
^{13}C NMR (100MHz, $CDCl_3$, δ ppm): 171.17, 170.63, 134.17, 134.15, 104.58, 103.90, 73.85, 73.78, 68.66, 68.13, 65.35, 65.29, 33.03, 32.48, 32.32, 31.44, 30.57, 30.55, 19.04, 13.65.
HR-ESI-MS $C_{14}H_{19}NO_6$ 297.12124 found 297.12116

ethyl 2-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)-1,3-dioxolane-4-carboxylate, mixture of *cis*- and *trans*-isomers.
^{1}H NMR (400MHz, $CDCl_3$, δ ppm): 6.67 (s, 4H, *cis* + *trans*), 5.11 (t, J = 3.7 Hz, 1H, *trans*), 5.04 (t, J = 4.3 Hz, 1H, *cis*), 4.56 – 4.52 (m, 1H, *trans*), 4.50 (dd, J = 7.5, 3.6 Hz, 1H, *cis*), 4.30 – 4.24 (m, 1H, *trans*), 4.21 (dd, J = 14.4, 7.3 Hz, 4H, *cis* + *trans*), 4.18 – 4.13 (m, 1H, *cis*), 4.01 (t, J = 8.1 Hz, 1H, *cis*), 3.85 – 3.79 (m, 1H, *trans*), 3.73 (dt, J = 6.7, 2.0 Hz, 2H, *cis*), 3.69 (t, J = 6.7 Hz, 2H, *trans*), 2.15 – 1.98 (m, 4H, *cis* + *trans*), 1.28 (t, J = 7.1 Hz, 6H, *cis* + *trans*).
^{13}C NMR (100MHz, $CDCl_3$, δ ppm): 171.08, 170.65, 170.58, 134.15, 104.58, 103.89, 73.83, 73.74, 68.63, 68.09, 61.49, 61.44, 33.00, 32.47, 32.27, 31.43, 14.17, 14.13.
HR-ESI-MS $C_{12}H_{15}NO_6$ 269.08994 found 269.08979

2-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)-1,3-dioxolane-4-carboxylic acid, 5c – (5-2)



Chemical Formula: $C_{10}H_{11}NO_6$
Molecular Weight: 241,20

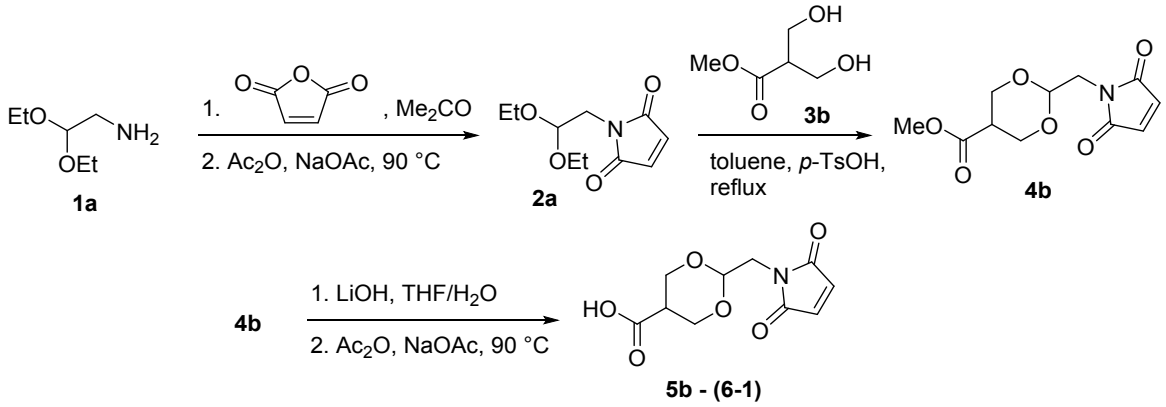
A solution of LiOH (2.5 eq., 50.7 mg, 0.0355 mL, 2.12 mmol) in water (1.41 mL) was poured to a solution of **4c** and ethyl 2-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)-1,3-dioxolane-4-carboxylate (in total 252 mg, 1 eq.) in THF (1.41 mL) and the reaction mixture was stirred at r.t. for 30 minutes. Completion of the reaction was checked by TLC, then EtOAc was added and the mixture was acidified with aqueous 3M solution of HCl to pH 2. The aqueous phase was extracted with EtOAc and the combined organic layers were washed with water and with brine, dried over Na_2SO_4 and concentrated to give the intermediate product used in the next step without further purification.
2-{2-[(2Z)-3-carboxyprop-2-enamido]ethyl}-1,3-dioxolane-4-carboxylic acid (1 eq., 130 mg, 0.502 mmol) was dissolved in acetic anhydride (6.78 mL) and sodium acetate (2.4 eq., 98.7 mg, 1.2 mmol) was added. The reaction mixture was stirred at 90 °C for 2 h and controlled by TLC. Acetic anhydride was evaporated under reduced pressure and 5 mL of water was added to the residue. The mixture was stirred at r.t. for 30 min and then extracted with ethyl acetate. After solvent evaporation the resulting crude material was purified by preparative HPLC to afford **5c** as a light-yellow oil in 54% overall yield (mixture of c*is* and *trans*-isomers).

^{1}H NMR (400MHz, MeOH-d4, δ ppm): 6.81 (s, 4H, *cis* + *trans*), 5.08 (s, 1H, *trans*), 5.02 (s, 1H, *cis*), 4.57 (t, J = 7.6 Hz, 1H, *trans*), 4.53 (d, J = 3.8 Hz, 1H, *cis*), 4.30 (t, J = 8.0 Hz, 1H, *trans*), 4.15 (d, J = 8.0 Hz, 1H, *cis*), 4.06 (t, J = 8.1 Hz, 1H, *cis*), 3.84 (t, J = 7.2 Hz, 1H, *trans*), 3.77 – 3.63 (m, 4H, *cis* + *trans*), 2.09 – 1.96 (m, 4H, *cis* + *trans*).

^{13}C NMR (100MHz, MeOH-d4, δ ppm): 173.26, 172.74, 171.07, 134.08, 134.05, 104.26, 103.62, 73.36, 68.22, 67.80, 32.50, 32.00, 31.81, 31.08.

HR-ESI-MS $C_{10}H_{11}NO_6$ 241.05864 found 241.05785

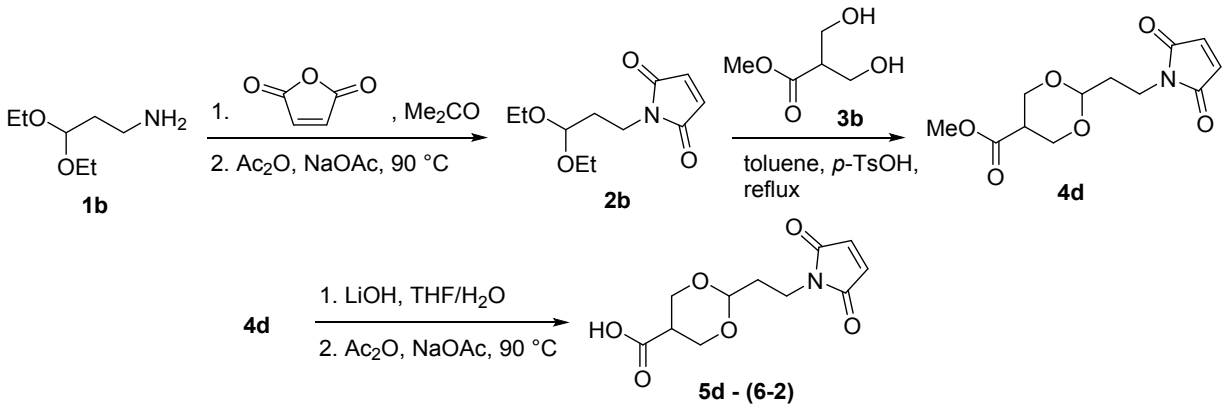
Synthesis of linker (6-1)



Scheme S3: synthesis of linker **6-1**

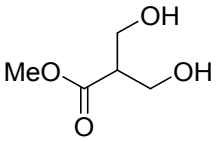
The linker **5b – (6-1)** was synthesized according to the reported procedures.[1]

Synthesis of linker (6-2)



Scheme S4: synthesis of linker 6-2

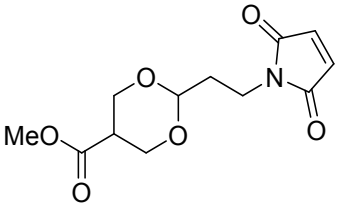
methyl 3-hydroxy-2-(hydroxymethyl)propanoate, 3b



Chemical Formula: $C_5H_{10}O_4$
Molecular Weight: 134,13

Molecule **3b** was synthetized according to the reported procedures.[1]

methyl 2-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)-1,3-dioxane-5-carboxylate, 4d



Chemical Formula: $C_{12}H_{15}NO_6$
Molecular Weight: 269,25

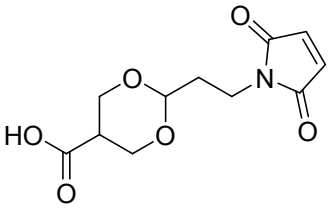
A solution of **2b** (1 eq., 500 mg, 2.2 mmol) and **3b** (0.895 eq., 264 mg, 1.97 mmol) in toluene (7.33 mL) containing catalytic amount of *p*-toluensulfonic acid monohydrate (0.2 eq., 83.7 mg, 0.44 mmol) was refluxed for 2 h. Ethanol was removed as azeotrope of toluene (b.p. of azeotrope: 76.7°C). After the reaction was complete, toluene was evaporated and the residue was dissolved in EtOAc and washed with a saturated solution of $NaHCO_3$ and with brine. The organic phase was dried over Na_2SO_4, filtrated and the solvent was evaporated at reduced pressure. The crude product was purified by flash chromatography (cyclohexane, then cyclohexane to EtOAc) to give **4d** (362 mg, 1.34 mmol, 68 %) as a yellow liquid (mixture of c*is* and *trans*-isomers).

^{1}H NMR (400MHz, $CDCl_3$, δ ppm): 6.67 (d, J = 3.6 Hz, 2H, *cis* + *trans*), 4.59 – 4.51 (m, 1H, *cis*), 4.49 (t, J = 4.7 Hz, 1H, *trans*), 4.24 (dd, J = 11.6, 4.6 Hz, 2H, *trans*), 3.84 (d, J = 10.3 Hz, 1H, *cis*), 3.79 (s, 1H, *cis*), 3.74 – 3.67 (m, 2H, *trans*), 3.68 – 3.57 (m, 5H, *cis* + *trans*), 3.03 – 2.88 (m, 1H, *trans*), 2.27 (s, 1H, *cis*), 1.94 – 1.82 (m, 2H, *cis* + *trans*).

^{13}C NMR (100MHz, $CDCl_3$, δ ppm): 170.68, 170.24, 134.16, 134.13, 100.87, 100.15, 67.50, 66.83, 52.35, 51.83, 39.90, 39.77, 33.37, 33.20, 33.16, 33.08.

HR-ESI-MS $C_{12}H_{15}NO_6$ 269.08994 found 269.09001

2-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)-1,3-dioxane-5-carboxylic acid, 5c – (5-2)



Chemical Formula: $C_{11}H_{13}NO_6$
Molecular Weight: 255,23

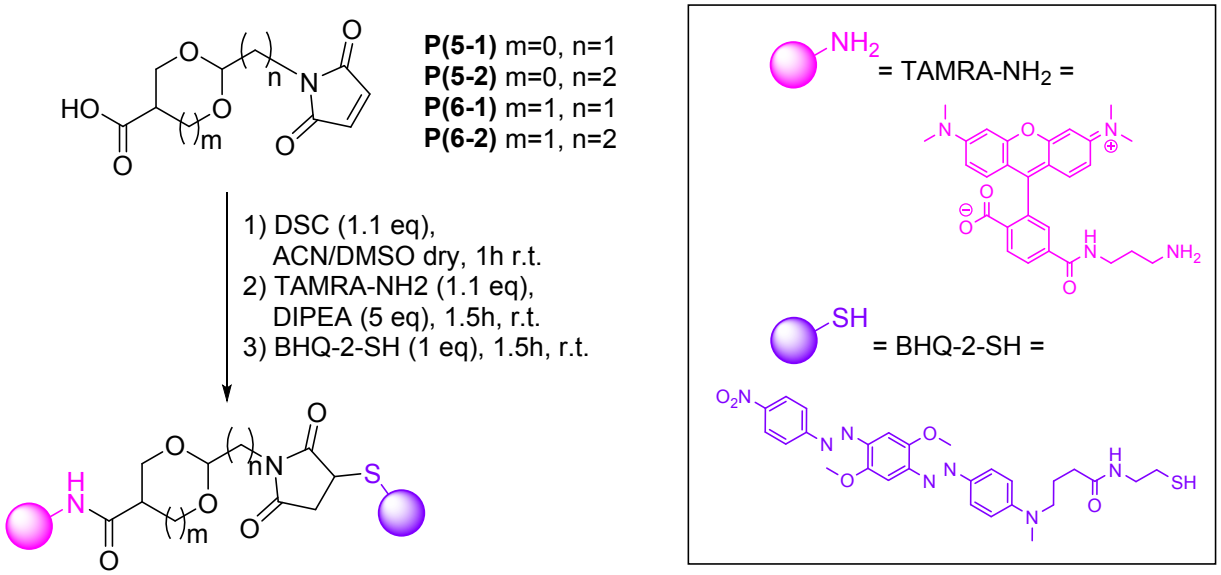
Using the similar procedure as for **5a**, the linker **5c** was synthesized as a light-yellow oil in 79% overall yield (mixture of c*is* and *trans*-isomers).

^{1}H NMR (400MHz, MeOH-d4, δ ppm): 6.75 (s, 2H, *trans*), 6.74 (s, 2H, *cis*), 4.57 (t, J = 5.0 Hz, 1H, *cis*), 4.48 (t, J = 4.8 Hz, 1H, *trans*), 4.41 (d, J = 10.7 Hz, 2H, *cis*), 4.17 (dd, J = 11.8, 4.8 Hz, 2H, *trans*), 3.87 – 3.81 (m, 2H, *cis*), 3.67 (t, J = 11.6 Hz, 2H, *trans*), 3.56 (dt, J = 13.9, 6.9 Hz, 4H, *cis* + *trans*), 2.90 – 2.81 (m, 1H, *trans*), 2.32 – 2.29 (m, 1H, *cis*), 1.80 (d, J = 4.9 Hz, 2H, *trans*), 1.76 (td, J = 6.9, 5.0 Hz, 2H, *cis*).

^{13}C NMR (100MHz, MeOH-d4, δ ppm): 171.07, 134.06, 134.03, 100.59, 100.03, 67.38, 66.63, 39.66, 39.04, 33.01, 32.66, 32.60.

HR-ESI-MS $C_{11}H_{13}NO_6$ 255.07429 found 255.071341

Synthesis of FRET probes P(5-1), P(5-2), P(6-1) and P(6-2)



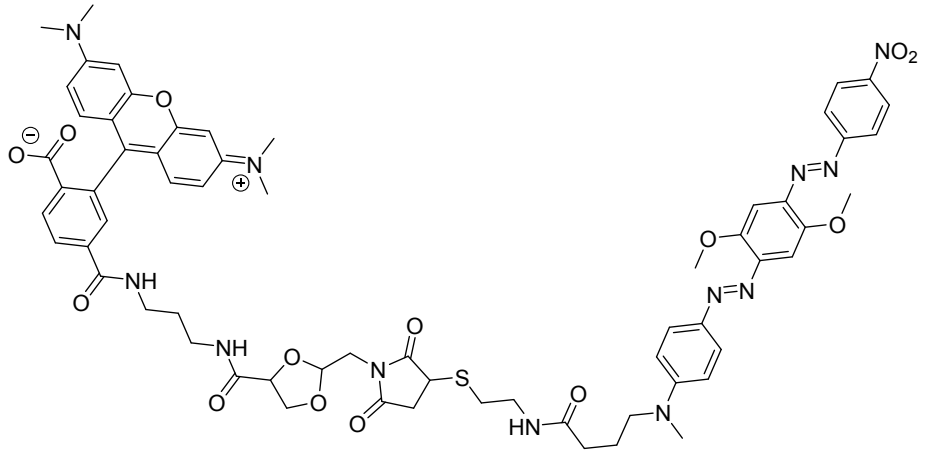
Scheme S5: general synthesis of FRET probes

Genaral procedure for synthesis of FRET probes

Solutions of starting acid (1 eq., 1 mg/100µL, in dry DMSO) and DIPEA (1 eq., 0.1 M in dry ACN) were mixed under Argon at room temperature, after few minutes N,N′-Disuccinimidyl carbonate (1.2 eq) was added and the reaction mixture was stirred for 1 hour. Then TAMRA-NH_2 (1.05 eq., 0.1 M in dry ACN) was added and the reaction was let to stir, checking with LC-MS the formation of TAMRA-ACID adduct. If after 1h, no product was detected, 5eq of DIPEA were added (to neutralize TFA molecules coming from HPLC purification of TAMRA-NH_2). When the intermediary product was formed, BHQ-2-SH (1 eq., 0.025 M in dry DMSO) and DIPEA (5 eq., 0.1 M in dry ACN) were added and let to stir until formation of the product was detected with LC-MS. The reaction mixture was then purified by preparative HPLC to give the product as a violet solid.

P(5-1)

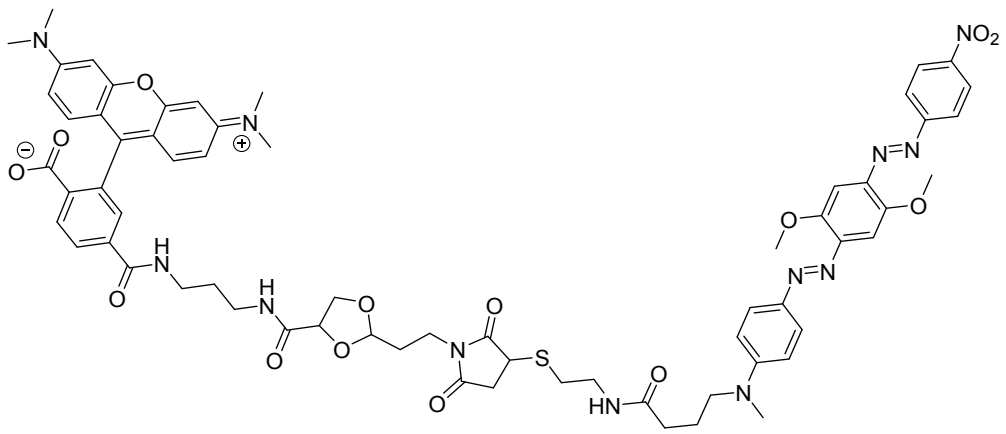
4-((3-(2-((3-((2-(4-((4-((E)-(2,5-dimethoxy-4-((E)-(4-nitrophenyl)diazenyl)phenyl)diazenyl)phenyl) (methyl)amino)butanamido)ethyl)thio)-2,5-dioxopyrrolidin-1-yl)methyl)-1,3-dioxolane-4-carbo-xamido)propyl)carbamoyl)-2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)benzoate



Yield 55% **1H NMR** (400 MHz, DMSO-d6) δ 8.78 (s, 1H), 8.44 (d, J = 8.1 Hz, 2H), 8.27 (d, J = 17.9 Hz, 2H), 8.06 (d, J = 7.7 Hz, 3H), 7.92 (s, 1H), 7.80 (d, J = 8.5 Hz, 2H), 7.39 (d, J = 26.4 Hz, 2H), 7.02 (s, 3H), 6.96 – 6.82 (m, 4H), 5.28 (s, 1H), 5.07 (t, 1H), 4.51 – 4.40 (m, 2H), 4.18 – 4.11 (m, 2H), 4.11 – 4.02 (m, 3H), 3.99 (s, 3H), 3.93 (s, 3H), 3.24 (s, 12H), 3.06 (s, 3H), 2.17 (s, 2H), 1.86 – 1.77 (m, 1H), 1.77 – 1.62 (m, 1H), 1.29 – 1.21 (m, 1H). **HR-ESI-MS** $C_{64}H_{68}N_{12}O_{14}S$ 1260.46987 found 1260.4714

P(5-2)

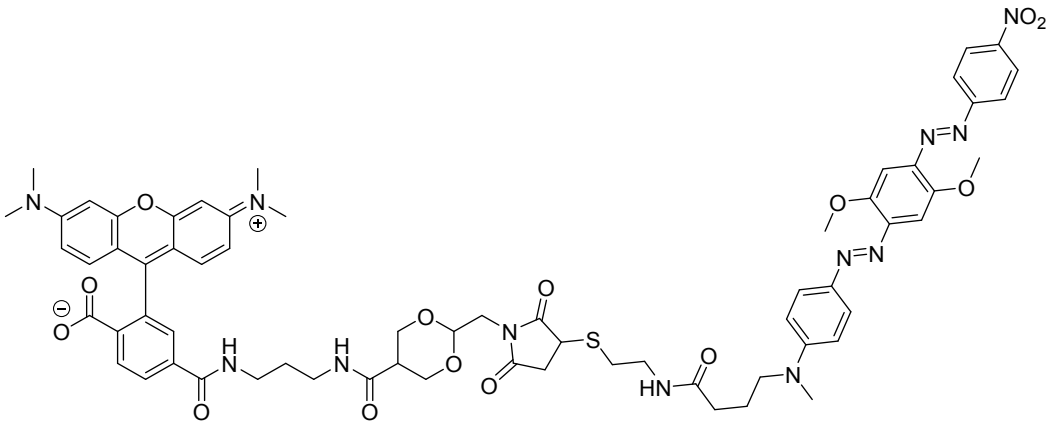
4-((3-(2-(2-(3-((2-(4-((4-((E)-(2,5-dimethoxy-4-((E)-(4-nitrophenyl)diazenyl)phenyl)diazenyl)phenyl)(methyl)amino)butanamido)ethyl)thio)-2,5-dioxopyrrolidin-1-yl)ethyl)-1,3-dioxolane-4-carboxamido)propyl)carbamoyl)-2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl) benzoate



Yield 63% **1H NMR** (400 MHz, DMSO-d6) δ 8.77 (s, 1H), 8.44 (d, J = 8.8 Hz, 2H), 8.25 (t, J = 12.4 Hz, 2H), 8.06 (d, J = 8.8 Hz, 2H), 7.91 (s, 1H), 7.80 (d, J = 8.8 Hz, 2H), 7.39 (d, J = 26.2 Hz, 2H), 7.02 (s, 3H), 6.95 – 6.83 (m, 3H), 4.92 (d, J = 12.3 Hz, 1H), 4.46 – 4.33 (m, 1H), 4.23 – 4.16 (m, 1H), 3.99 (s, 3H), 3.93 (s, 3H), 3.69 – 3.62 (m, 1H), 3.24 (s, 12H), 3.17 (d, J = 6.7 Hz, 3H), 3.06 (s, 3H), 2.93 – 2.80 (m, 1H), 2.77 – 2.70 (m, 1H), 2.16 (t, J = 7.1 Hz, 2H), 1.96 – 1.87 (m, J = 22.0 Hz, 1H), 1.86 – 1.75 (m, 2H), 1.74 – 1.62 (m, 2H), 1.24 (s, 2H). **HR-ESI-MS** $C_{65}H_{70}N_{12}O_{14}S$, 1274.48552; found 1274.48542.

P(6-1)

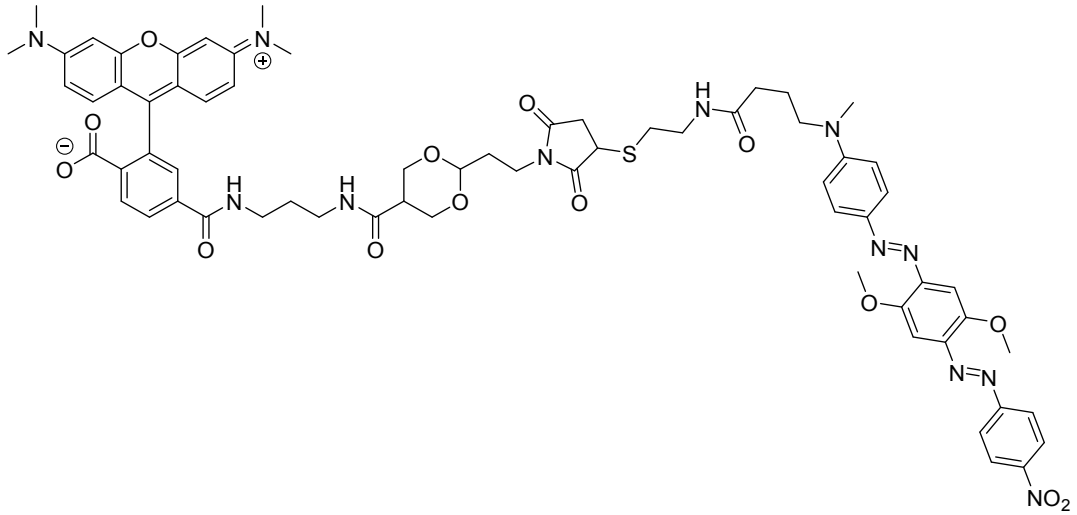
4-((3-(2-((3-((2-(4-((4-((E)-(2,5-dimethoxy-4-((E)-(4-nitrophenyl)diazenyl)phenyl)diazenyl)phenyl)(methyl)amino)butanamido)ethyl)thio)-2,5-dioxopyrrolidin-1-yl)methyl)-1,3-dioxane-5-carboxamido)propyl)carbamoyl)-2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl) benzoate



Yield 76% **1H NMR** (400MHz, DMSO-d6) δ 8.81 (t, *J*=5.3 Hz, 1 H), 8.43 (d, *J*=8.8 Hz, 2 H), 8.29 - 8.33 (m, 1 H), 8.24 - 8.29 (m, 1 H), 8.00 - 8.12 (m, 3 H), 7.95 (s, 1 H), 7.80 (d, *J*=9.0 Hz, 2 H), 7.72 (t, *J*=5.4 Hz, 1 H), 7.42 (s, 1 H), 7.36 (s, 1 H), 7.03 (s, 4 H), 6.91 (s, 2 H), 6.87 (d, *J*=9.0 Hz, 2 H), 4.75 (t, *J*=5.1 Hz, 1 H), 4.29 (d, *J*=11.5 Hz, 2 H), 4.02 - 4.07 (m, 1 H), 3.99 (s, 3 H), 3.94 (s, 3 H), 3.81-3.84 (m, 2H), 3.44 - 3.51 (m, 4 H), 3.30 - 3.36 (m, 4 H), 3.24 (s, 12H), 3.20 (d, *J*=8.3 Hz, 2 H), 3.07 (s, 3 H), 2.84 (dt, *J*=13.0, 6.4 Hz, 1 H), 2.71 (dt, *J*=13.2, 6.7 Hz, 1 H), 2.55-2.57 (m, 2H), 2.34 (br.s, 1H), 2.18 (d, *J*=5.5 Hz, 2 H), 1.76 - 1.86 (m, 2 H), 1.66 - 1.76 (m, 2 H) **HR-ESI-MS:** $C_{65}H_{70}N_{12}O_{14}S$, 1274.48552; found 1274.48491.

P(6-2)

4-((3-(2-(2-(3-((2-(4-((4-((E)-(2,5-dimethoxy-4-((E)-(4-nitrophenyl)diazenyl)phenyl)diazenyl)phenyl)(methyl)amino)butanamido)ethyl)thio)-2,5-dioxopyrrolidin-1-yl)ethyl)-1,3-dioxane-5-carboxamido)propyl)carbamoyl)-2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)benzoate



Yield 71% **1H NMR** (400 MHz, DMSO-d6) δ 8.75 (s, 2H), 8.44 (d, J = 8.7 Hz, 2H), 8.27 (d, J = 15.6 Hz, 2H), 8.06 (d, J = 8.4 Hz, 3H), 7.91 (s, 1H), 7.80 (d, J = 9.1 Hz, 2H), 7.64 (s, 1H), 7.39 (d, J = 26.4 Hz, 2H), 7.02 (s, 3H), 6.94 – 6.83 (m, 4H), 4.56 (s, 1H), 4.47 (s, 1H), 4.28 (d, J = 11.7 Hz, 2H), 3.99 (s, 4H), 3.93 (s, 3H), 3.80 (d, J = 10.4 Hz, 2H), 3.63 (t, J = 11.0 Hz, 1H), 3.24 (s, 9H), 3.07 (s, 4H), 2.86 (dd, J = 13.2, 6.7 Hz, 2H), 2.72 (dd, J = 13.3, 6.4 Hz, 2H), 2.21 – 2.12 (m, 3H), 1.80 (s, 3H), 1.68 (s, 5H), 1.24 (s, 1H). **HR-ESI-MS** $C_{66}H_{72}N_{12}O_{14}S$, 1288.50117; found 1288.49841

Stability of the FRET probes in aqueous media

Materials and methods

Aqueous buffers were prepared following Table S1, pH was measured with a pHmeter.

Calculated pH	Measured pH	Composition
0	<0,7	1 M solution of HCl
2	2,00	0,01 M solution of HCl
5	5,03	0,78 mL of Na_2HPO_4 0,1 M + 39,4 mL of NaH_2PO_4
7,4	7,32	30,96 mL of Na_2HPO_4 0,1 M + 9,04 mL of NaH_2PO_4
9	9,03	0,485 g of TRIS base in 15 mL of miliQ water, pH adjusted to 9 with 1M solution of HCl and then diluted with miliQ water to 40 mL

Table S1 Preparation of buffer solutions for the stability tests.

Fluorescence measurements were done using 96-well plates black Nunclon Delta Surface from Thermo Scientific and a fluorometer Perkin Elmer VictorX2 2030 Multilabel Reader.

Stability tests in aqueous buffers

Working solutions (40 µL in DMSO) of FRET probes **P(5-1), P(5-2), P(6-1)** and **P(6-2)** were prepared. 25 µL of each working solution was added to 975 µL of aqueous media (final concentration 1 µM), vortexed and distributed onto 96-well plates (in triplicates). The instrument temperature was set to 25 °C and excitation/emission wavelengths were set to those for TAMRA (550/580 nm). The fluorescence

was measured every 3 minutes for 15 hours and normalized to the fluorescence of a solution of TAMRA-NH_2 (1 µM) and BHQ-2-SH (1 µM) in water (2.5 % DMSO, positive control). For stability test at pH < 1 the fluorescence of MCC-FRET probe was monitored in parallel. The obtained results are shown in Figure
S1.

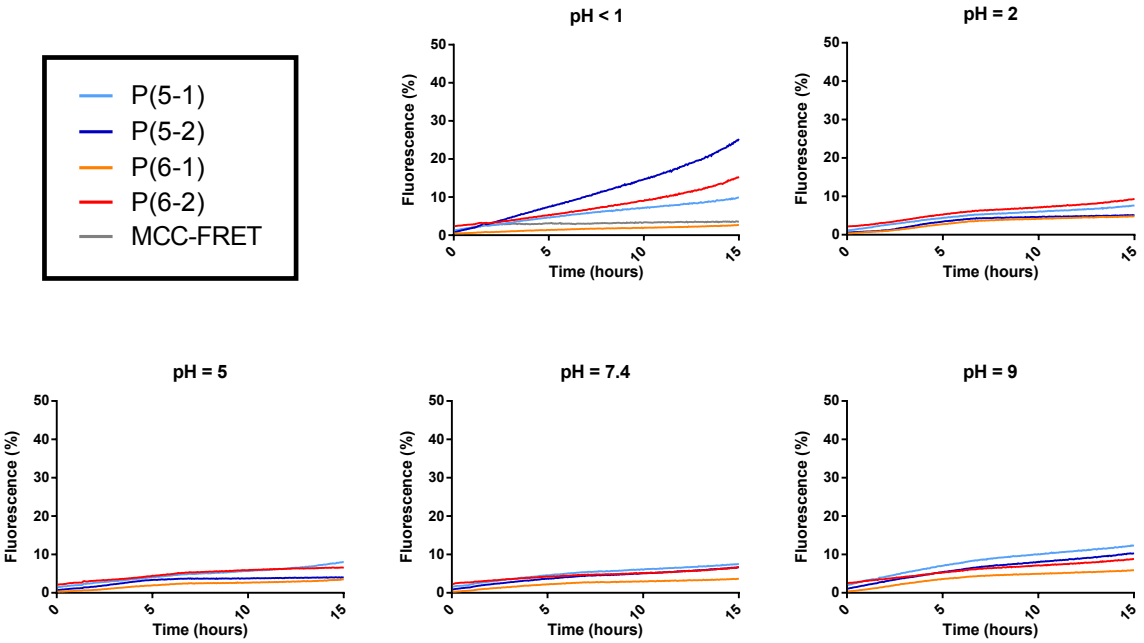


Figure S1 graphs of stability tests for all the FRET probes

Rate of succinimide ring-opening

Rate of succinimide ring-opening in PBS buffer

The solution (2 mL) of each FRET probes **P(5-1), P(5-2), P(6-1)** and **P(6-2)** (50 µM, final concentration) in PBS 1x buffer (pH 7.4 , DMSO 10%) was incubated at 37 ˚C. After certain intervals of time the aliquots (100 µL) were taken, diluted with 100 µL of acetonitrile and then were analysed by LC-MS. The conversion was calculated as the peak-area under hydrolyzed product (M+18) divided by the total peak-area. Results are shown in the main article.

Rate of succinimide ring-opening in human plasma

The solution (2 mL) of each FRET probes **P(5-1), P(5-2), P(6-1)** and **P(6-2)** (1 µM, final concentration) in human plasma (DMSO 10%) was incubated at 37 ˚C. After certain intervals of time 100 µL aliquots were taken and mixed with 100µL of acetonitrile, allowing the precipitation of proteins, the resulting mixture was centrifuged and the supernatant was analysed by LC-MS. The conversion was calculated as the area under opened product divided by the total area.

Human plasma was supplied by Etablissement Français du Sang (EFS Strasbourg).

Stability of FRET probes in human plasma

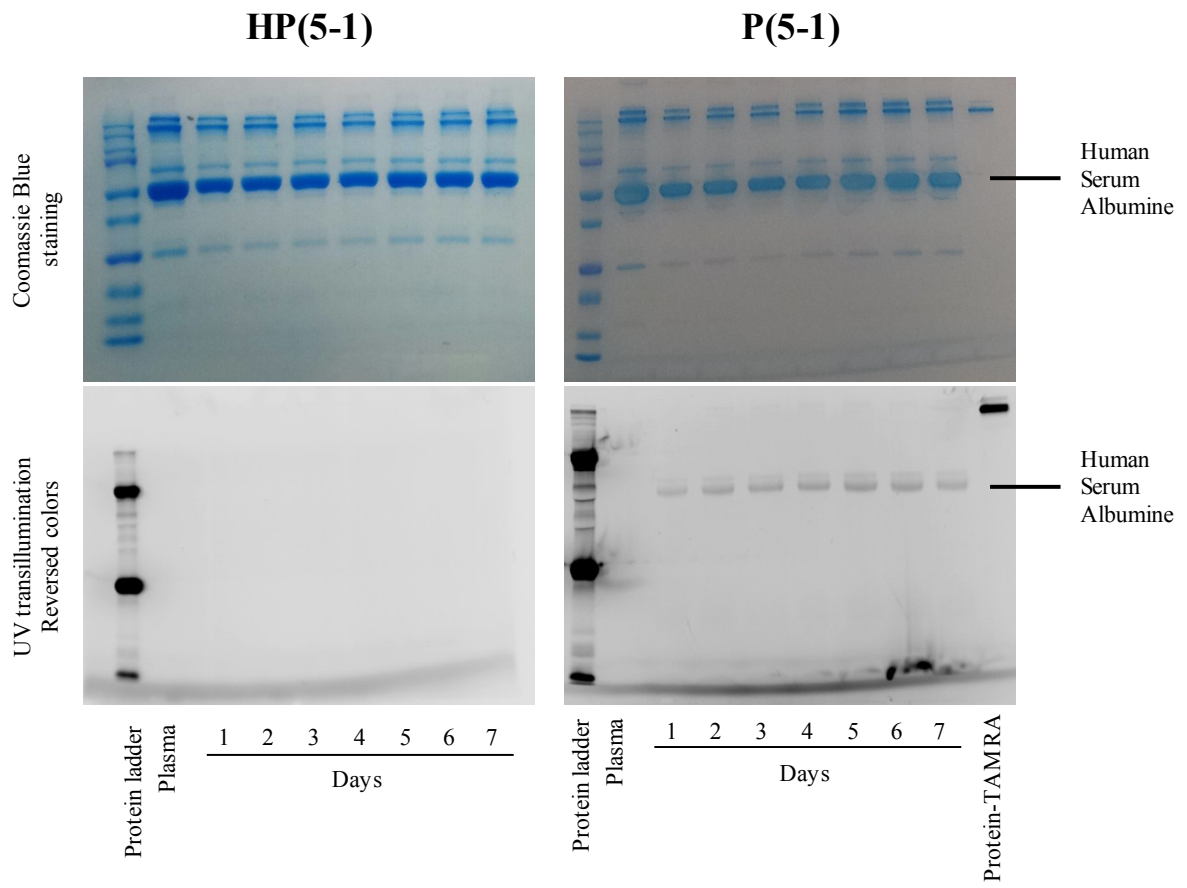
Procedure

1) Preparation of probe **HP(5-1)**.5 µL of 10 mM stock solution of FRET probe P(5-1) was added to 15 µL of PBS 1x buffer (pH 7.4) and 13.5 µL of DMSO in a vial for LC-MS (final concentration 0.5 mM). The resulting solution was incubated at 37 °C and analysed by LC-MS until 100% hydrolysis was achieved.

2) Preparation of probe **P(5-1).** Aliquots from 10 mM stock solutions of FRET probe P(5-1) was diluted to reach the final concentration of 0.5 mM (PBS/DMSO 1:1).

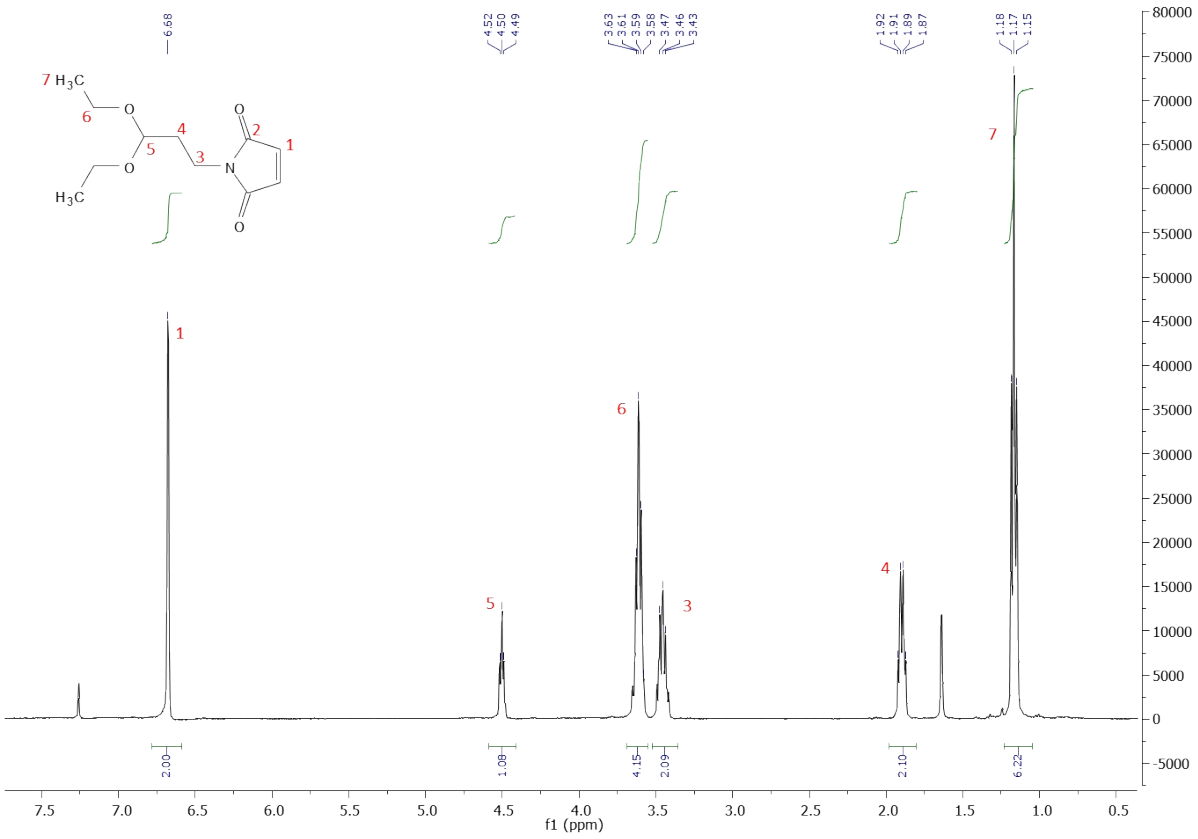
3) Incubation in human plasma. For every 0.5 mM solution of probes, 10 µL were taken and added to 90 µL of human plasma (final concentration of probes: 50 µM), the resulting solutions were incubated at 37 °C. Each day aliquots of 2 µL were taken, diluted 100 times with water and stocked at -20 °C.

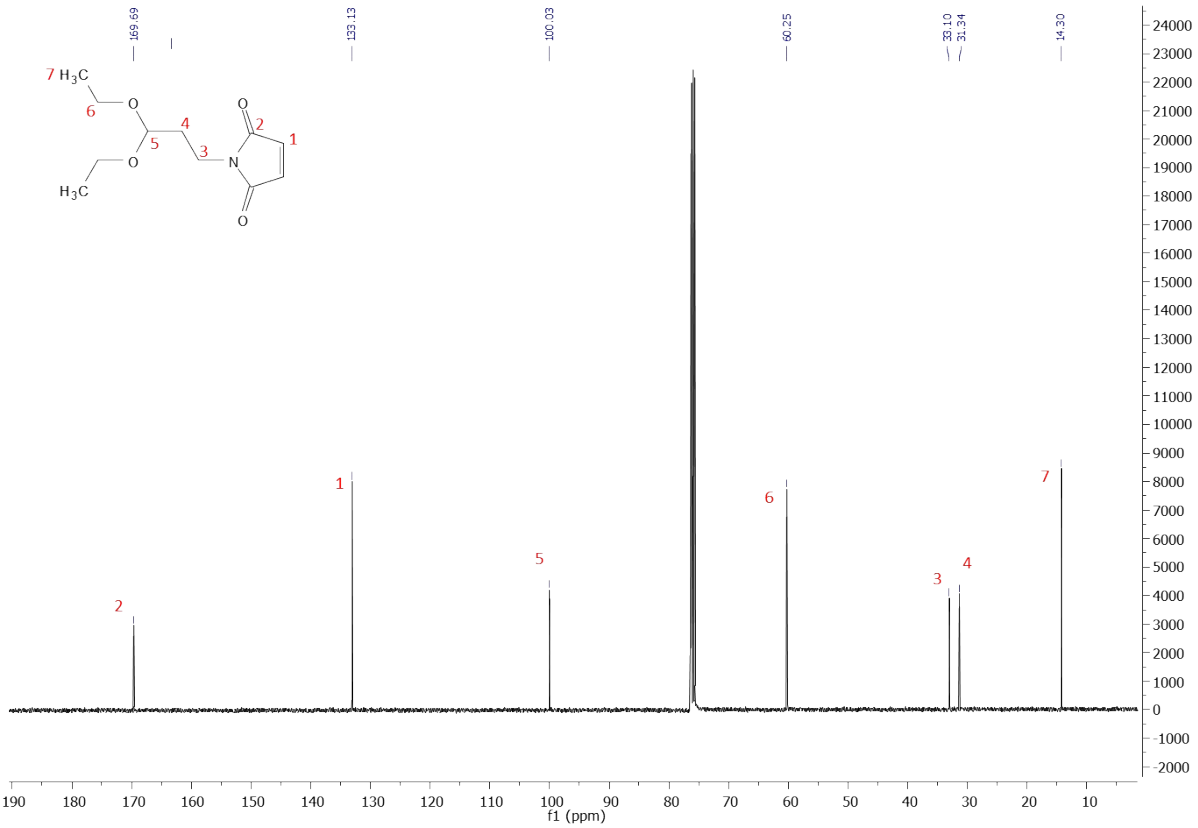
4) SDS-PAGE analysis Non-reducing SDS-PAGE was performed on 12% Mini-PROTEAN® TGX™ Gel (Bio-Rad ref 4561044) following standard lab procedures. For each solution of samples (including neat plasma diluted 10 times with water and a 0,1 mg/mL solution of antibody-TAMRA conjugate (standard control, with average degree of conjugation of 0,86) 24 µL of aliquot was taken and mixed with 8 µL of 4x non-reducing Laemmli SDS sample buffer (ref J63615, Alfa Aesar). The samples were heated at 95 °C for 5 minutes and loaded into the gel well (10 µL). The gel was run at constant voltage (200 V) for 40 min using TRIS 0.25 M - Glycine 1.92 M - SDS 1% as a running buffer. Fluorescence was measured on GeneGenius bio-imaging system (Syngene) and then normalized to standard control prior to staining with Coomassie Blue.



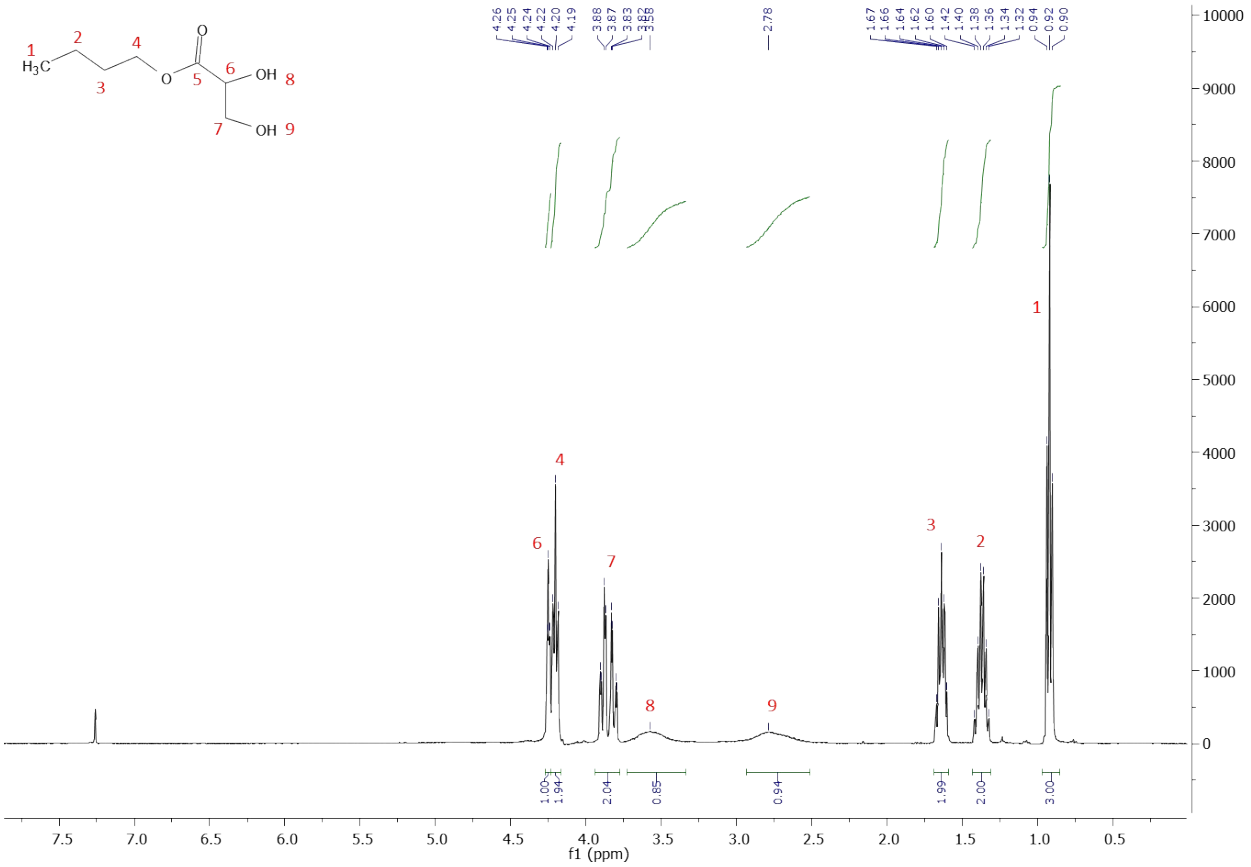
^{1}H and ^{13}C NMR spectra

Compound 2b





Compound 3a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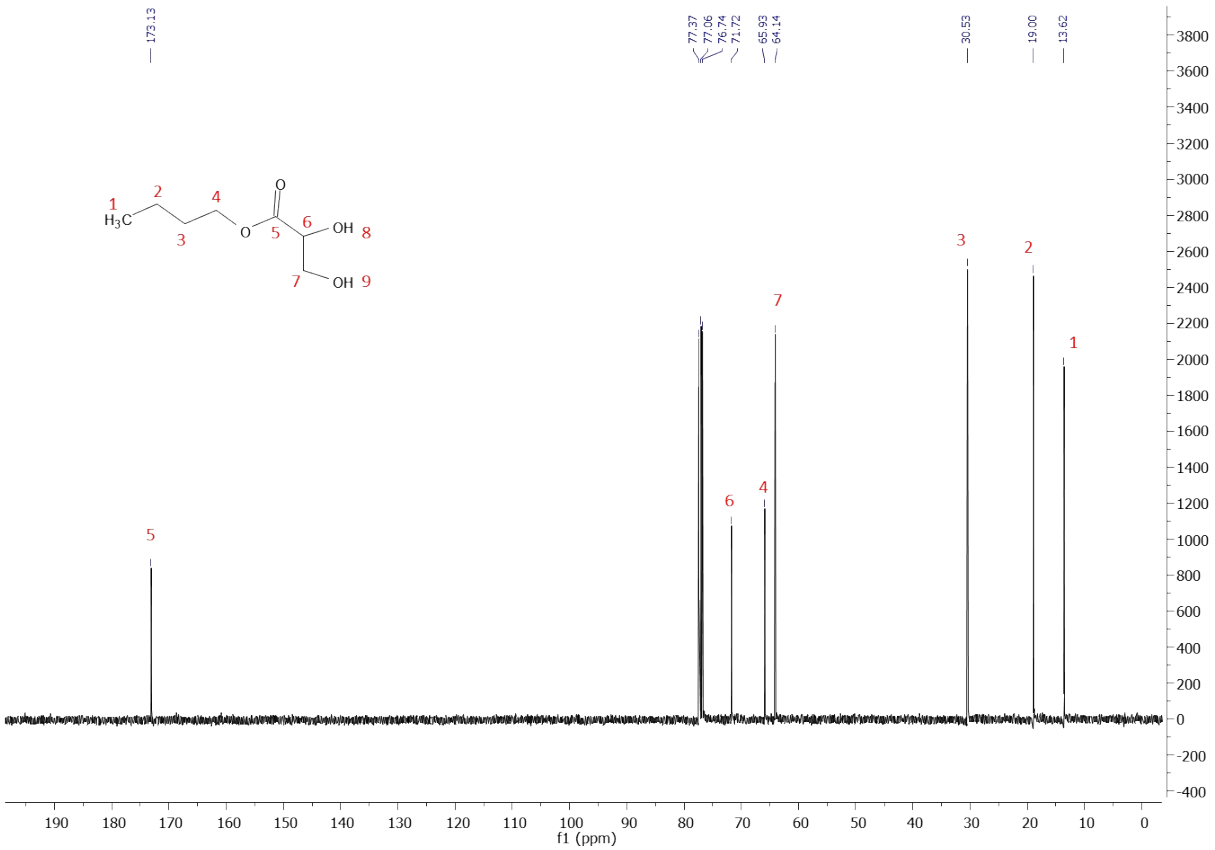
4.24
4.22
4.20
4.19
3.88
3.87
3.83
3.80
3.78
2.78
1.67
1.66
1.64
1.62
1.60
1.42
1.40
1.38
1.36
1.34
1.32
0.94
0.92
0.90
1.00
1.94
2.04
0.85
0.94
1.99
2.00
3.00
f1 (ppm)


173.13
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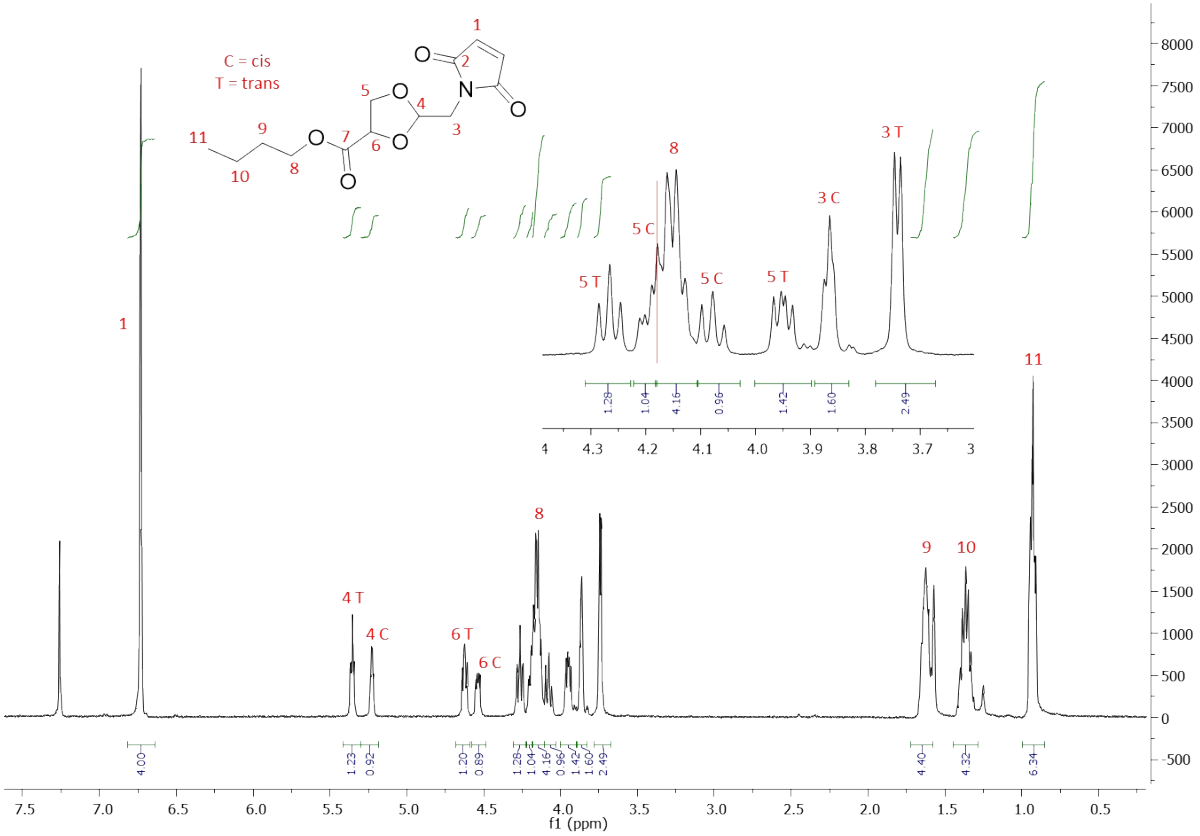
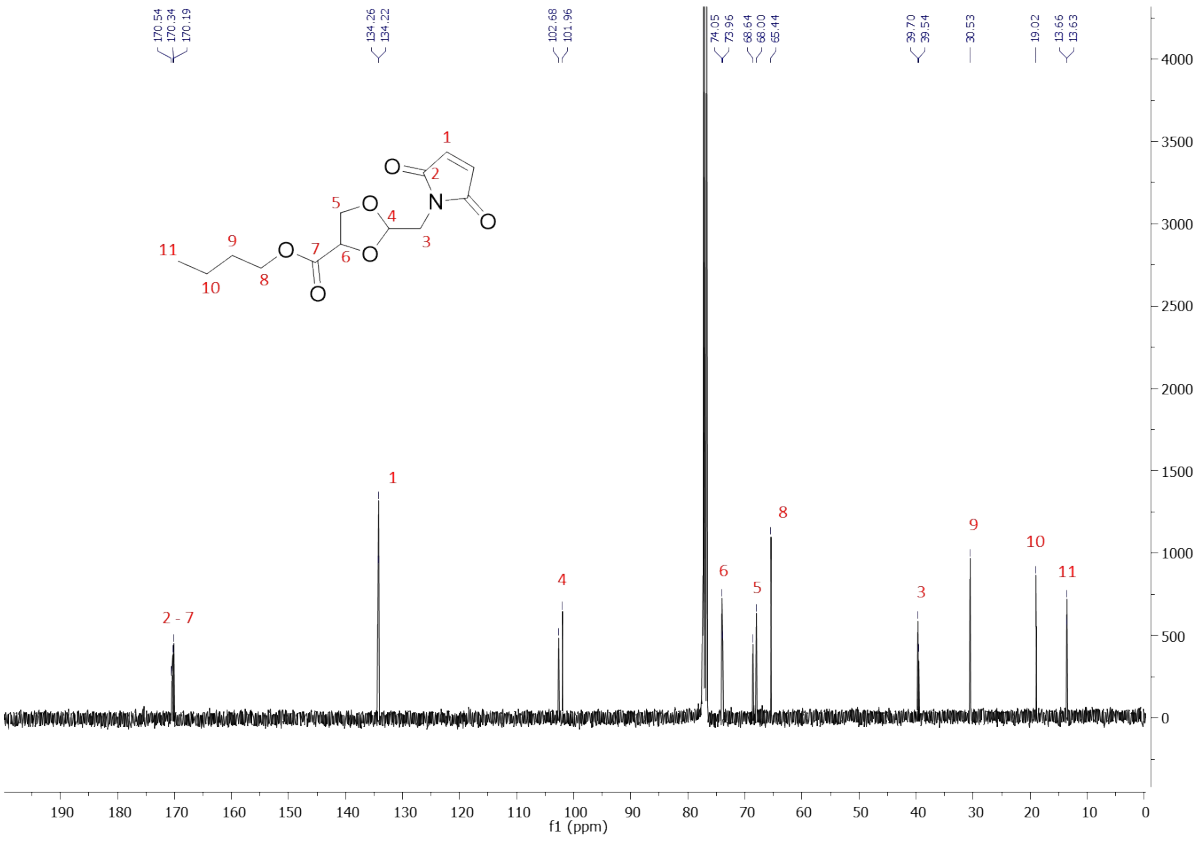
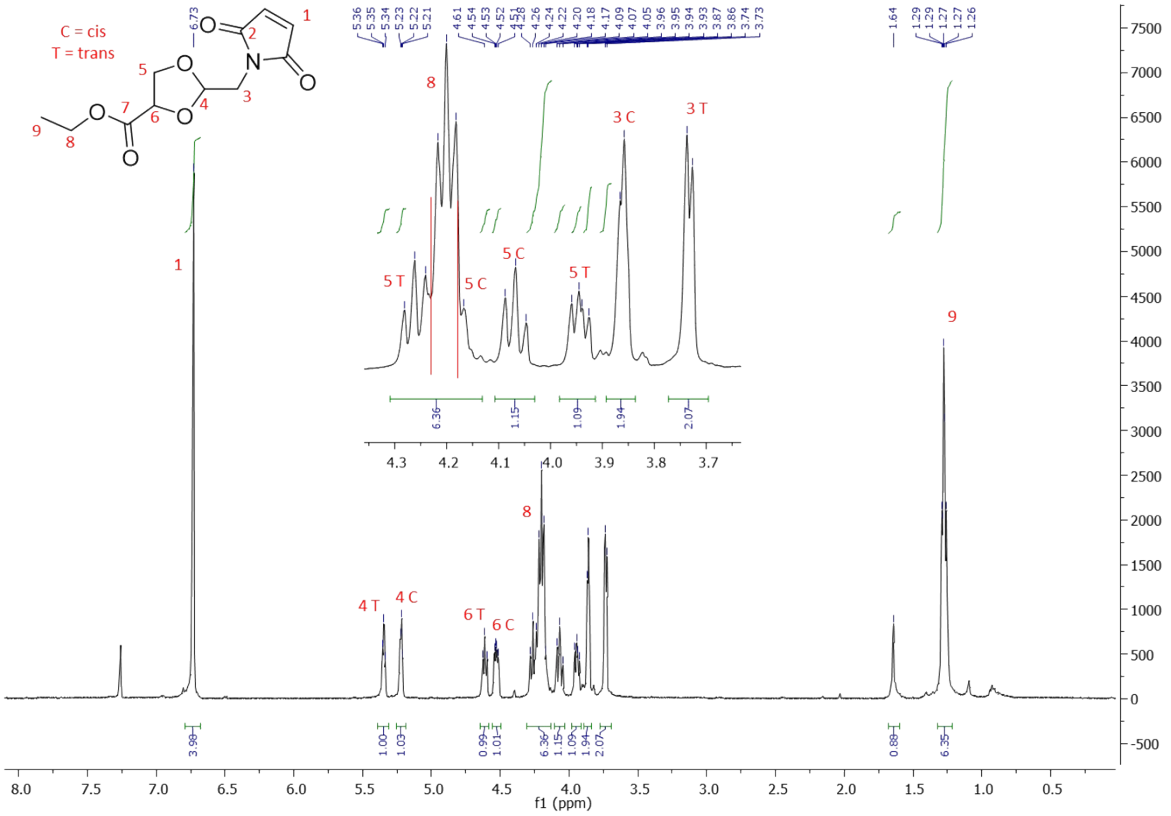
76.74
71.72
65.93
64.14
30.53
19.00
13.62
f1 (ppm)

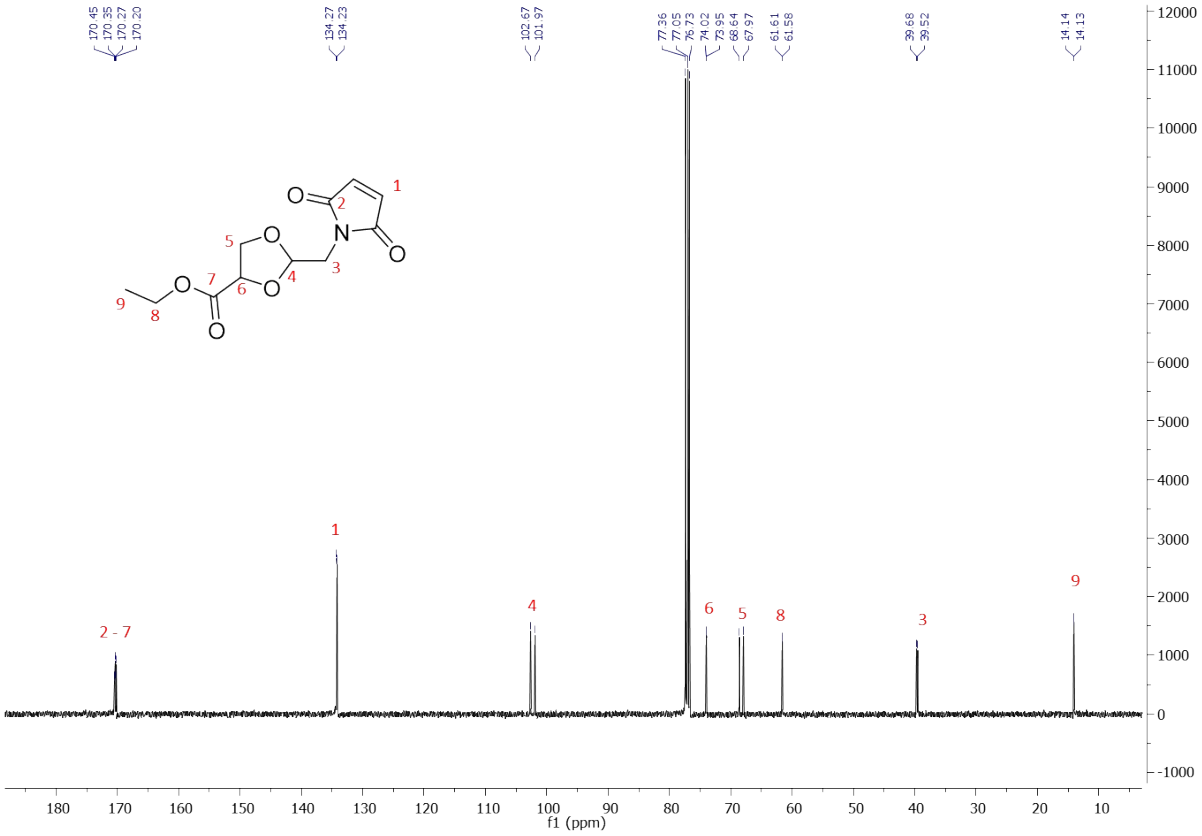
Compound 4a



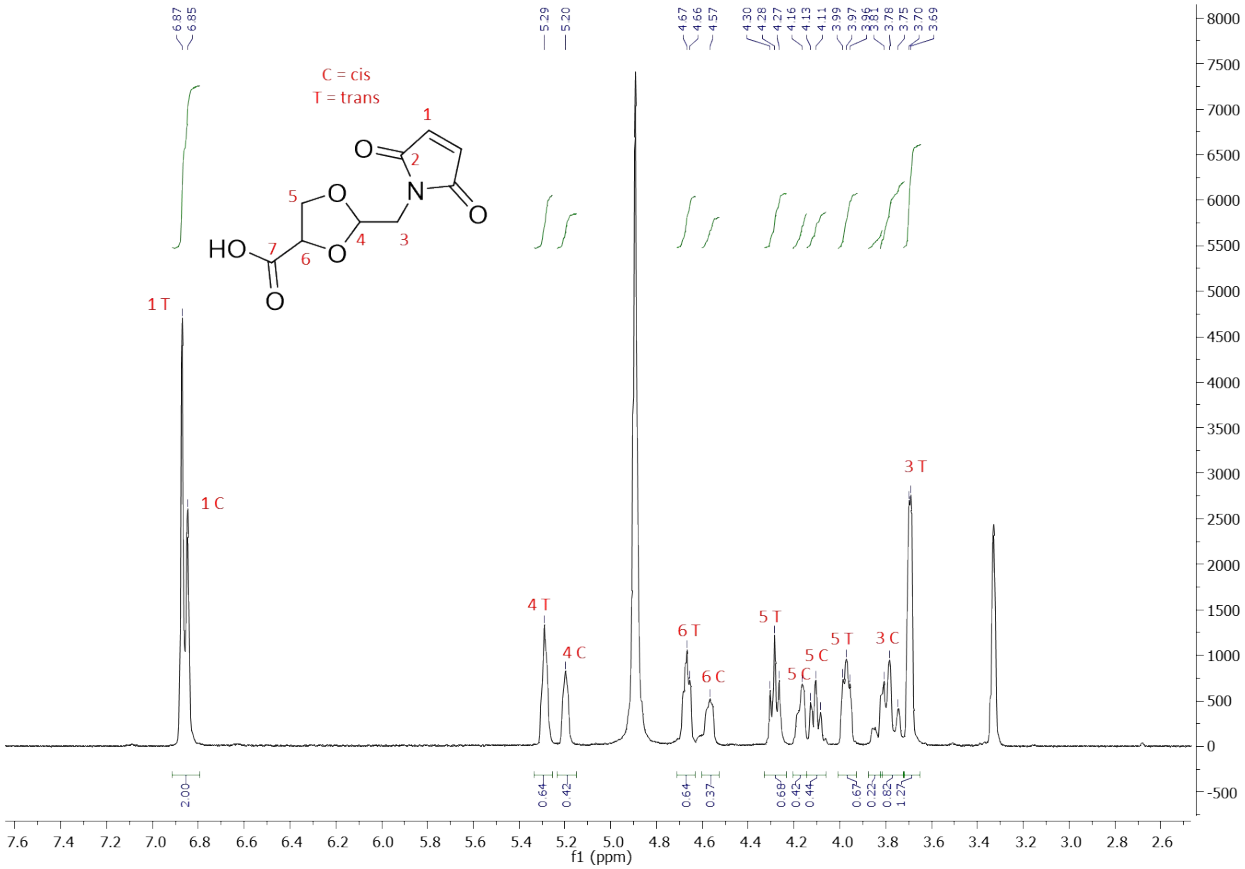


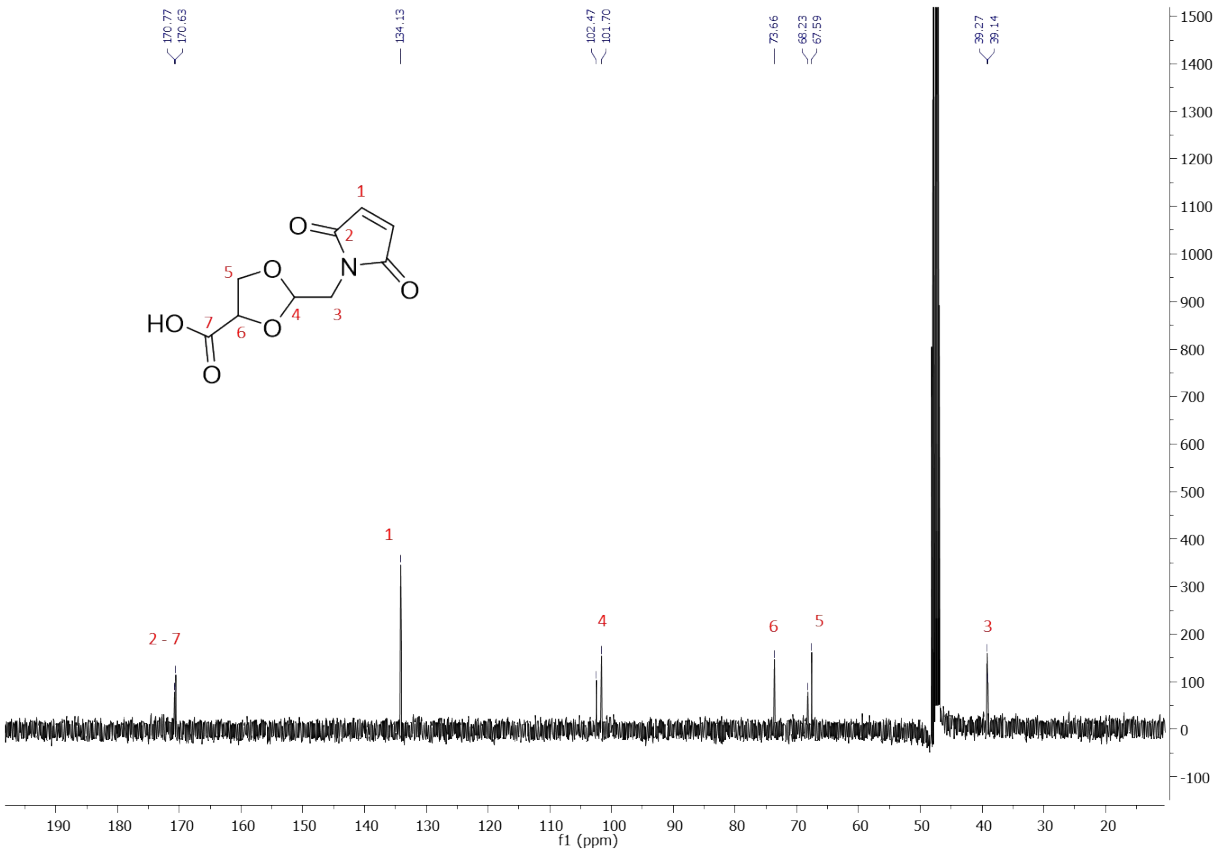
Compound **ethyl 2-((2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)methyl)-1,3-dioxolane-4-carboxylate**



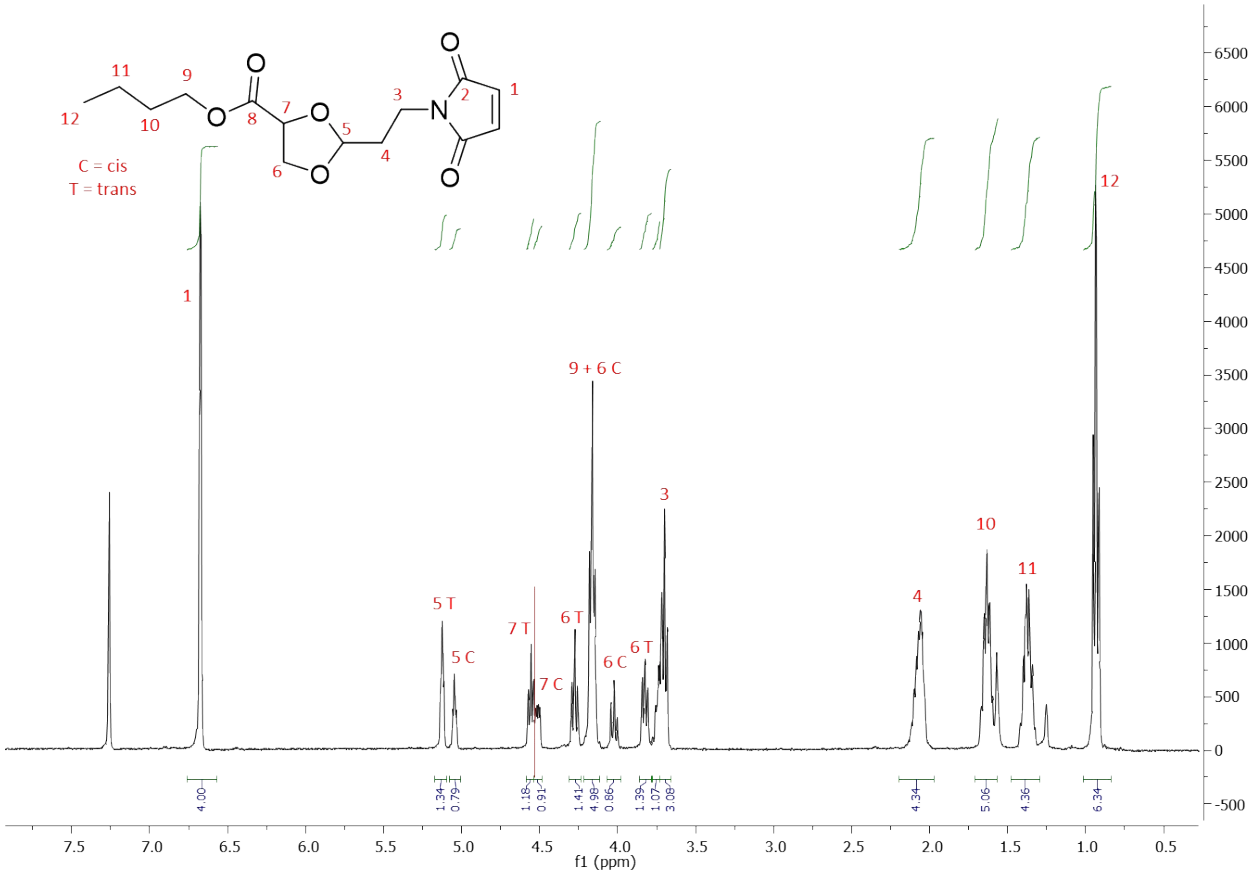


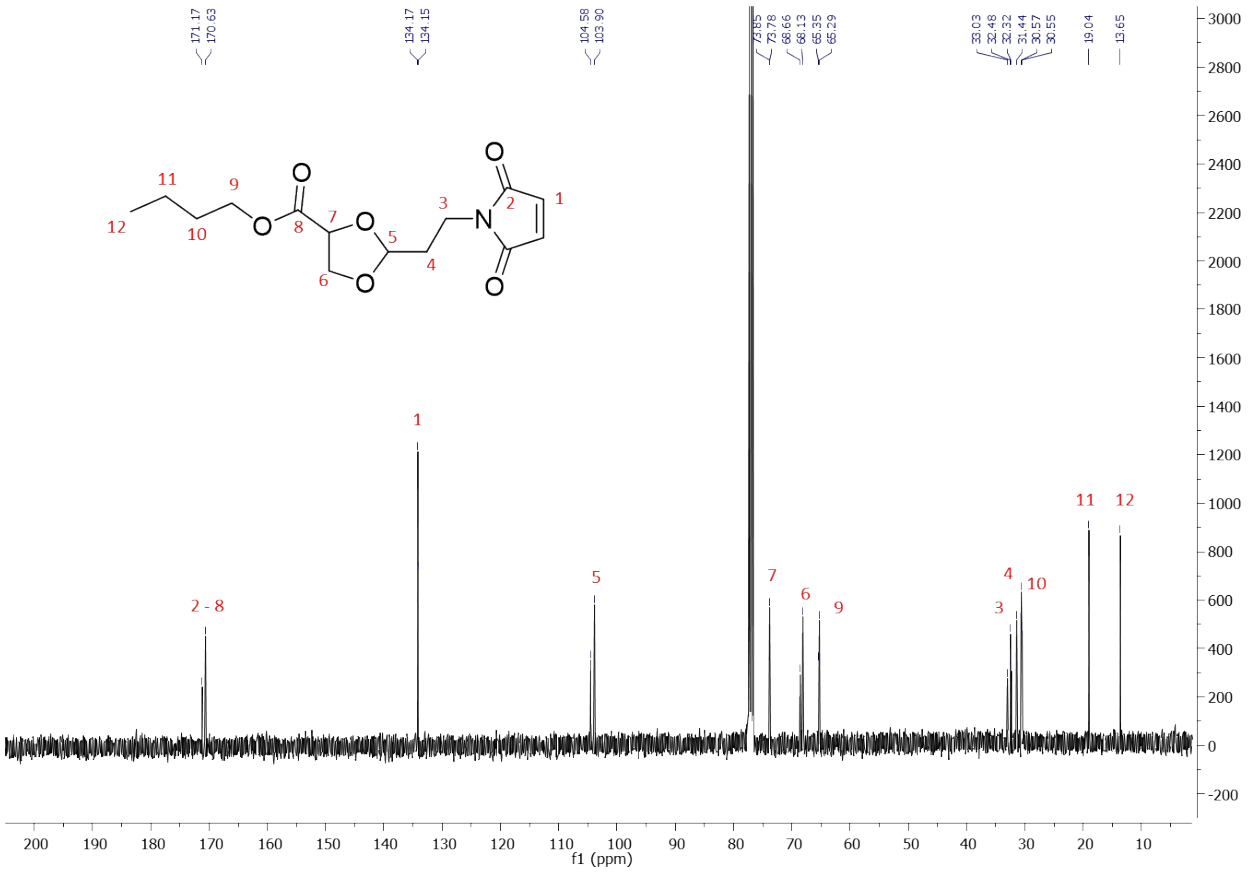
Compound 5a



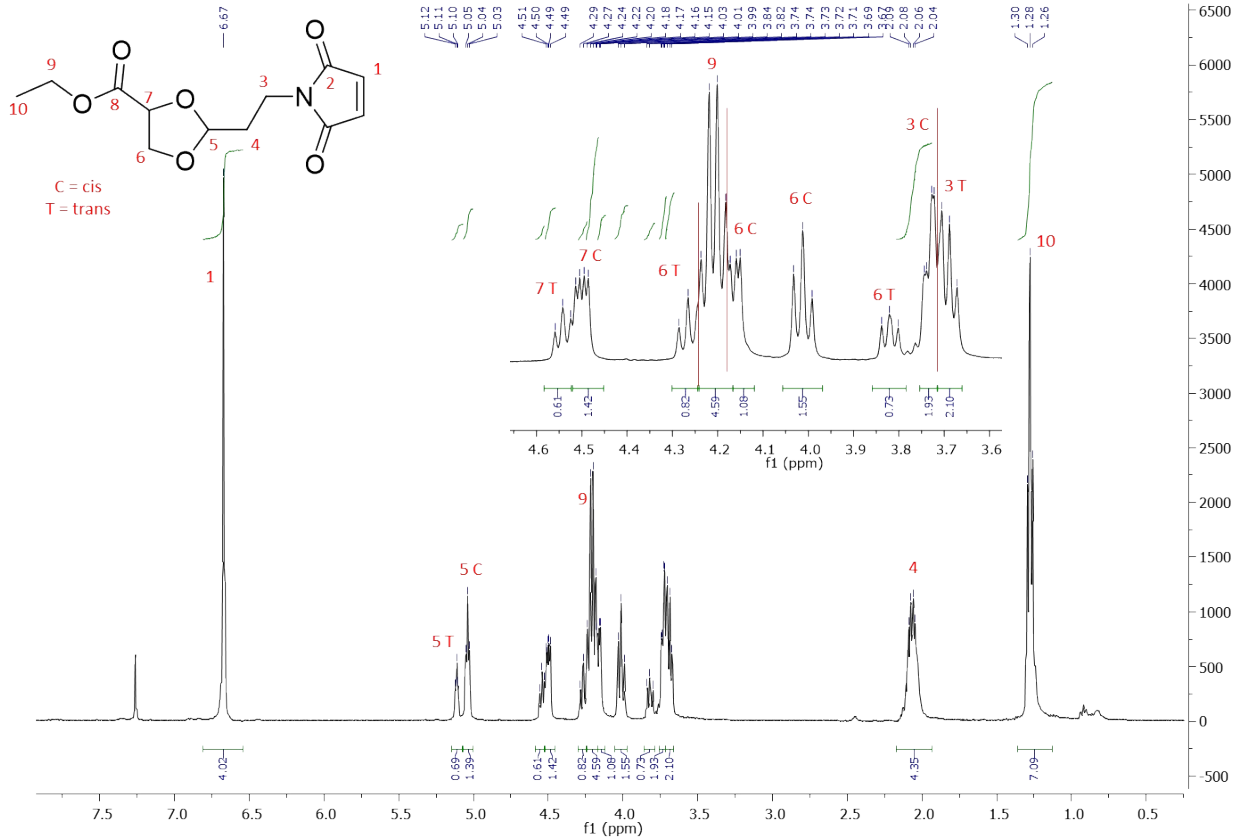


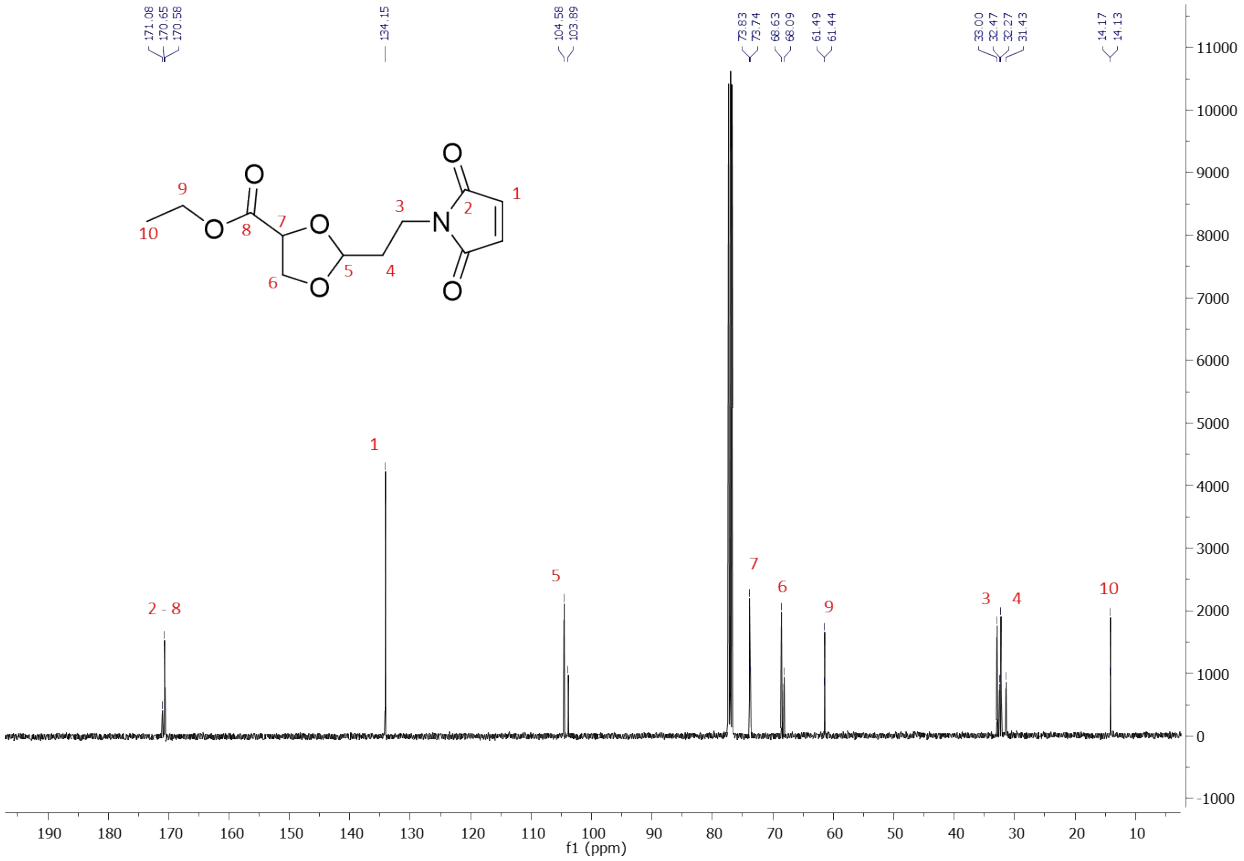
Compound 4c



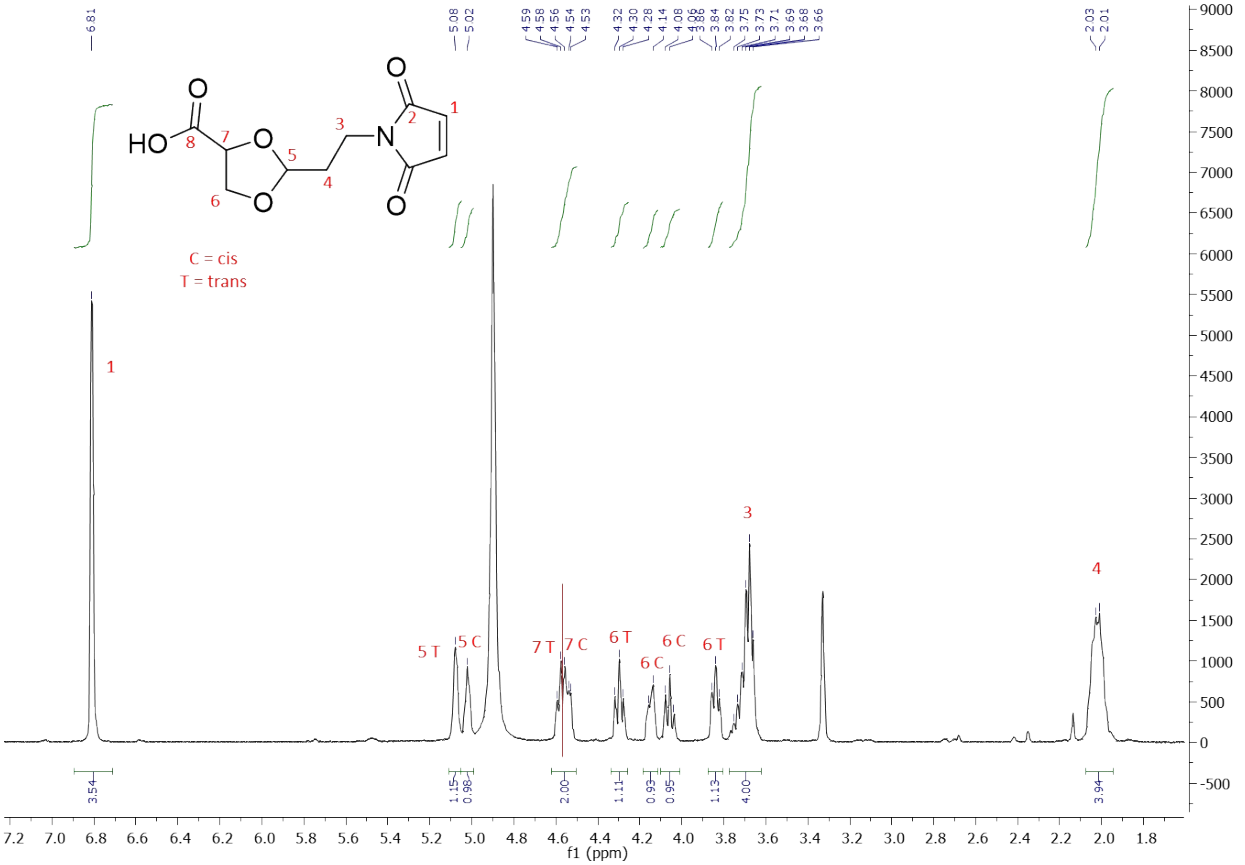


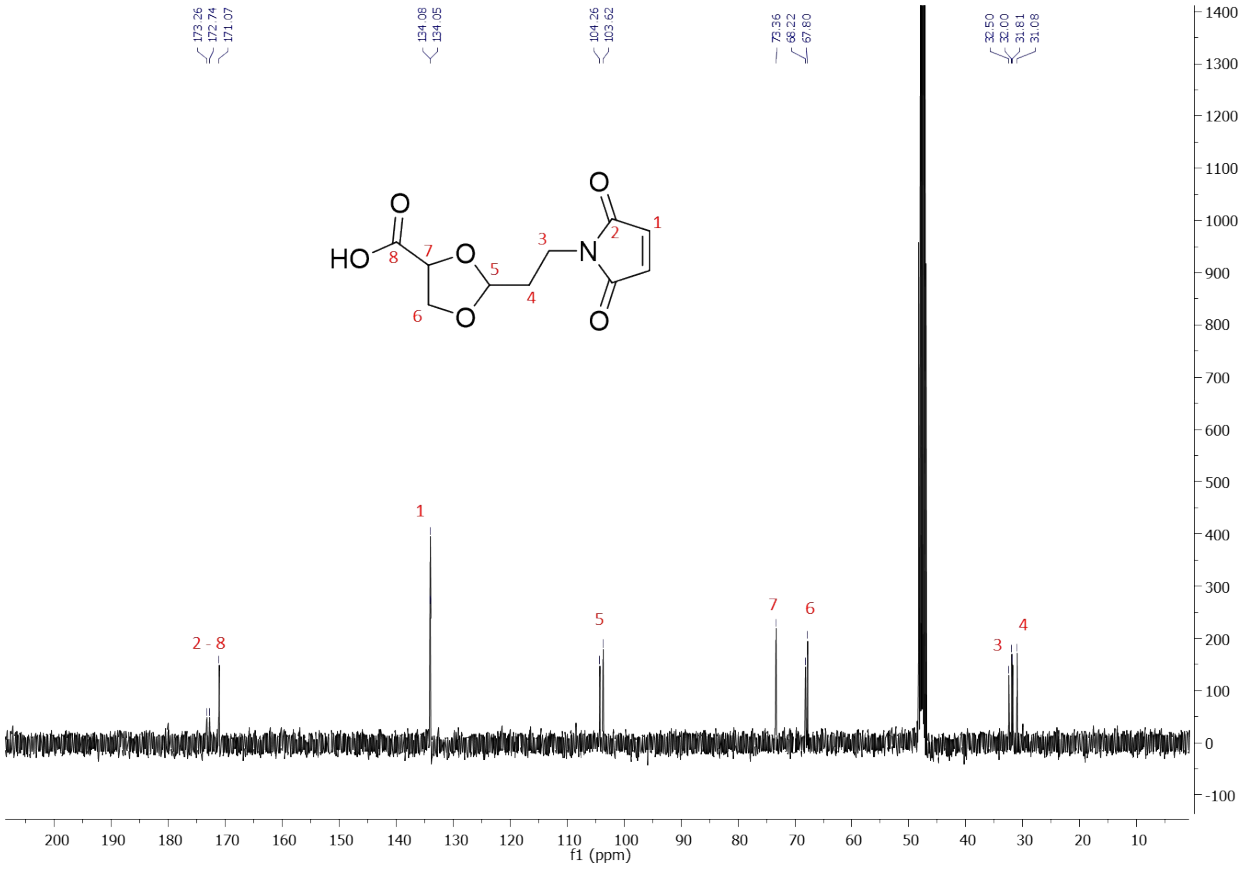
Compound **ethyl 2-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)-1,3-dioxolane-4-carboxylate**



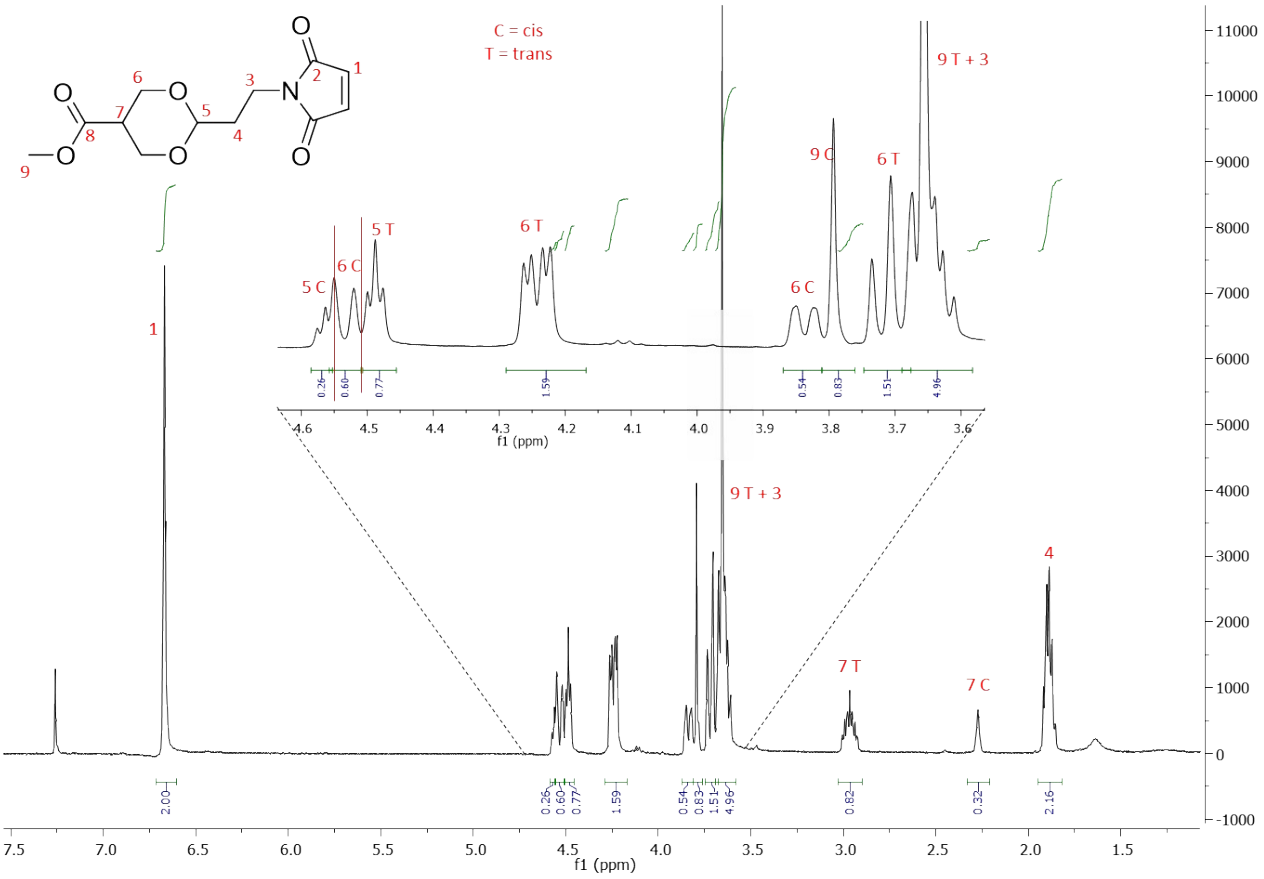


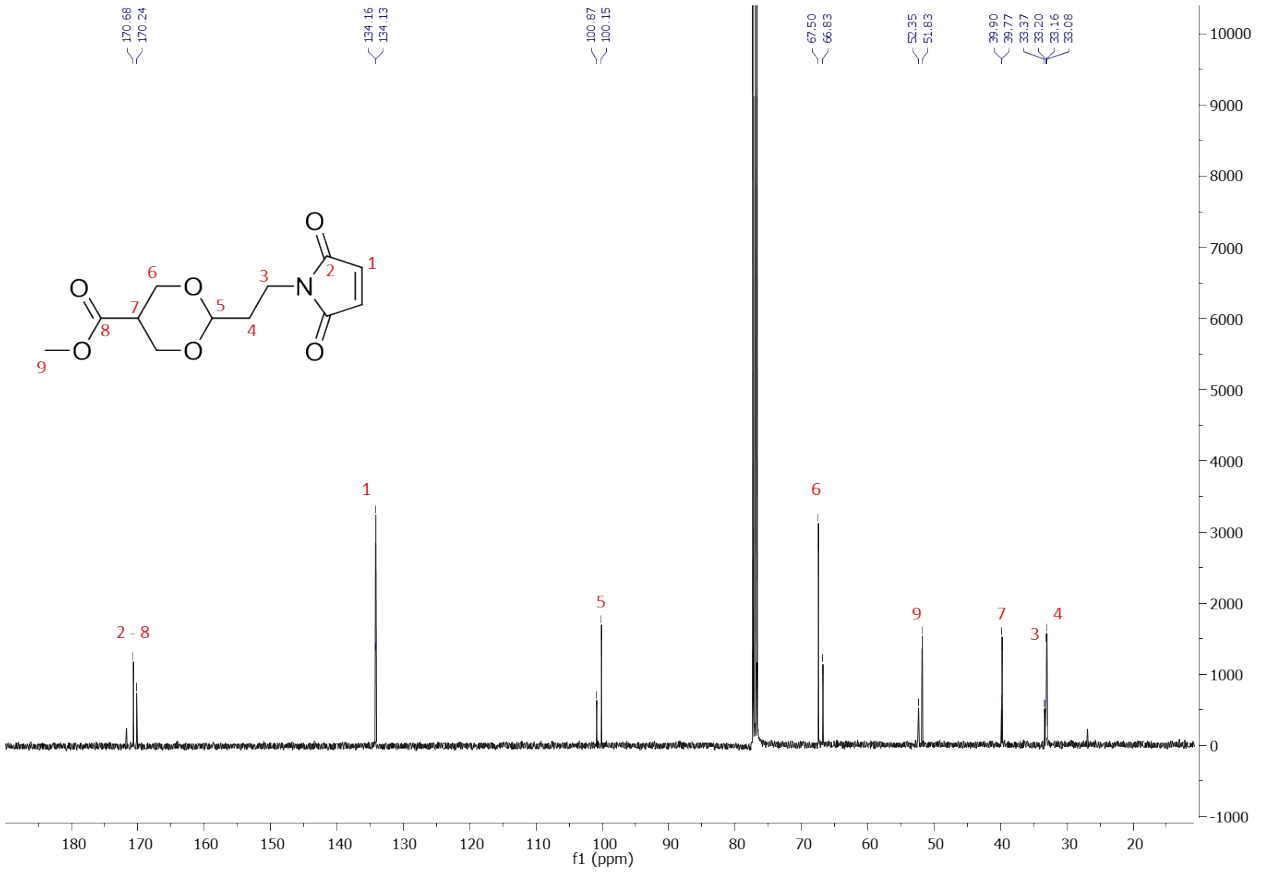
Compound 5c



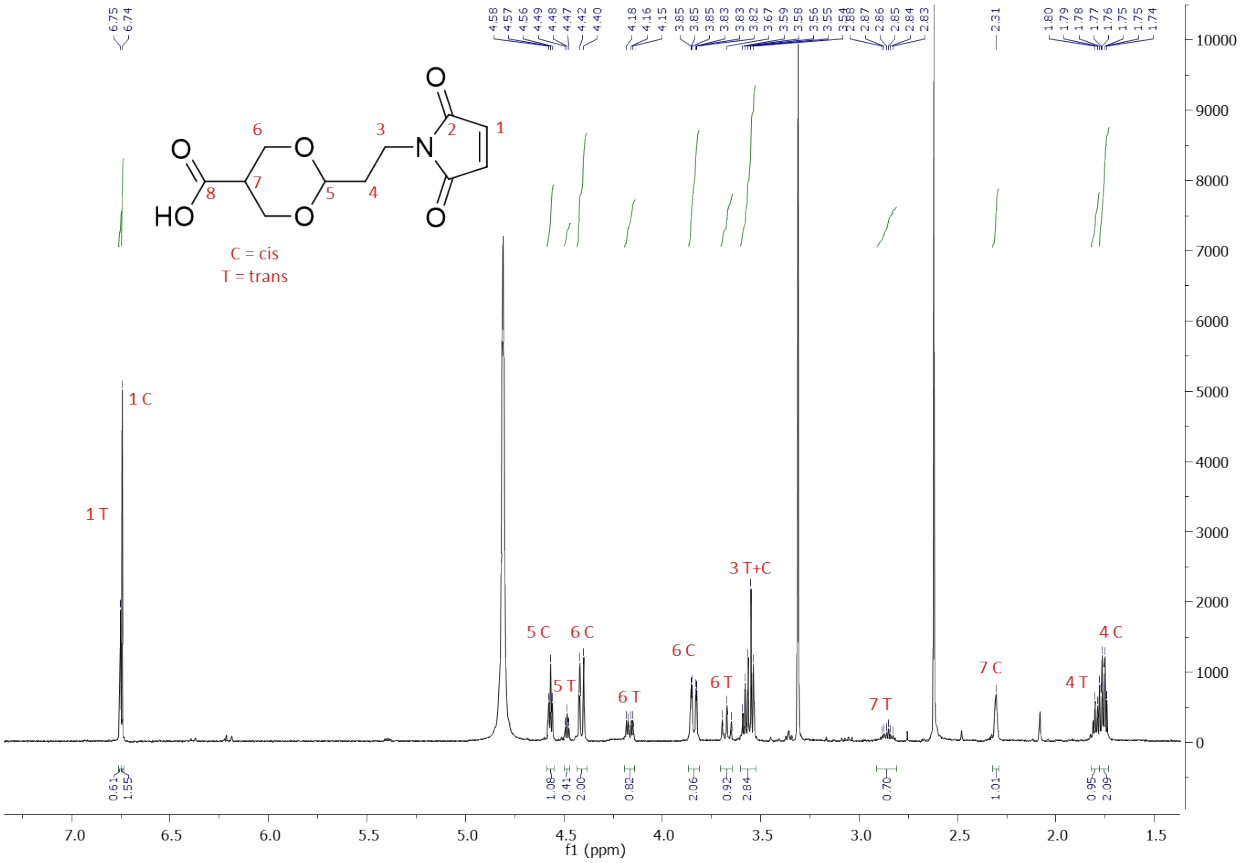


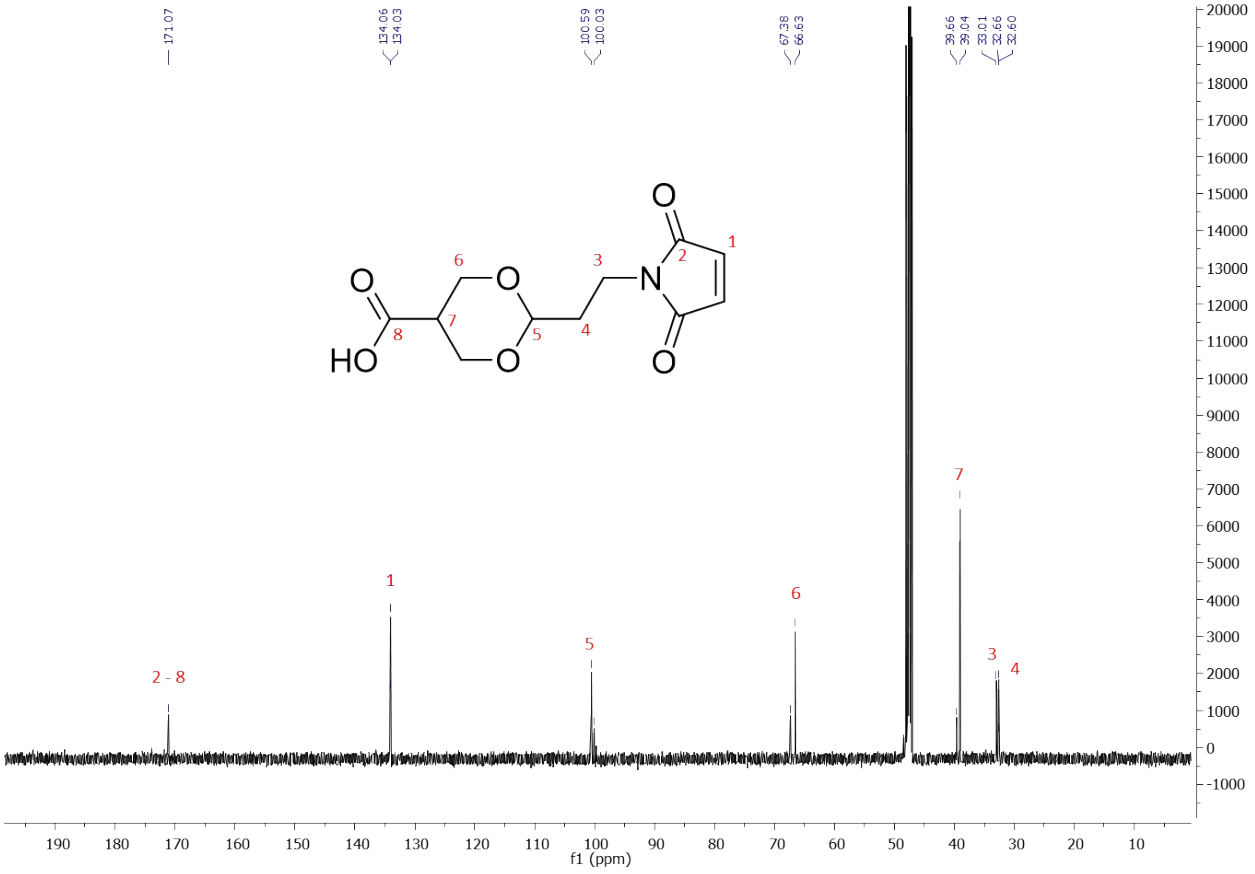
Compound 4d


C = cis
T = trans
9 T + 3
9 C
6 T
5 T
6 C
5 C
f1 (ppm)
7 T
7 C
4


170.68
170.24
134.16
134.13
100.87
100.15
67.50
66.83
52.35
51.83
39.90
39.77
33.37
33.20
33.16
33.08
2 - 8
f1 (ppm)

Compound 5d

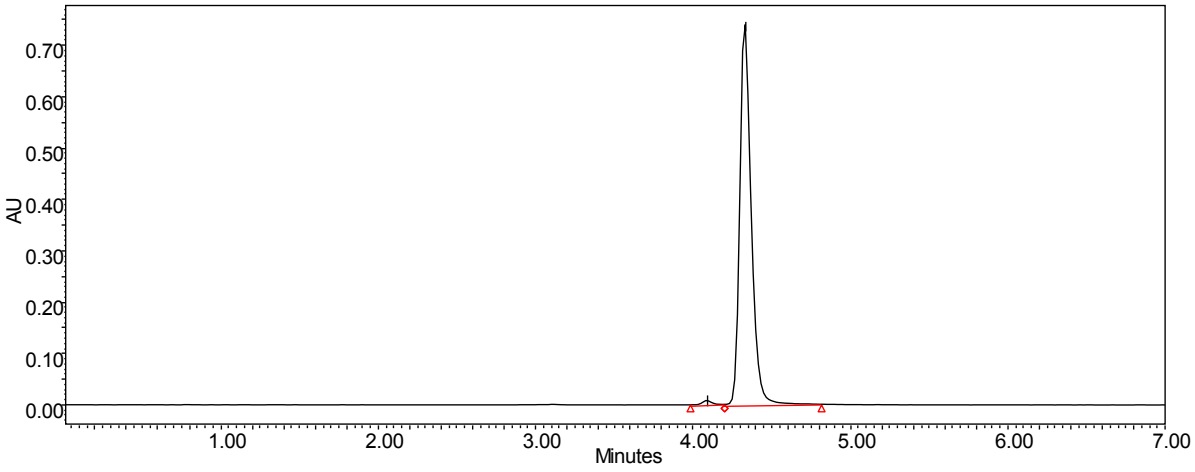

C = cis
T = trans
1 C
1 T
5 C
6 C
5 T
6 T
6 C
6 T
3 T+C
7 T
7 C
4 C
4 T
f1 (ppm)

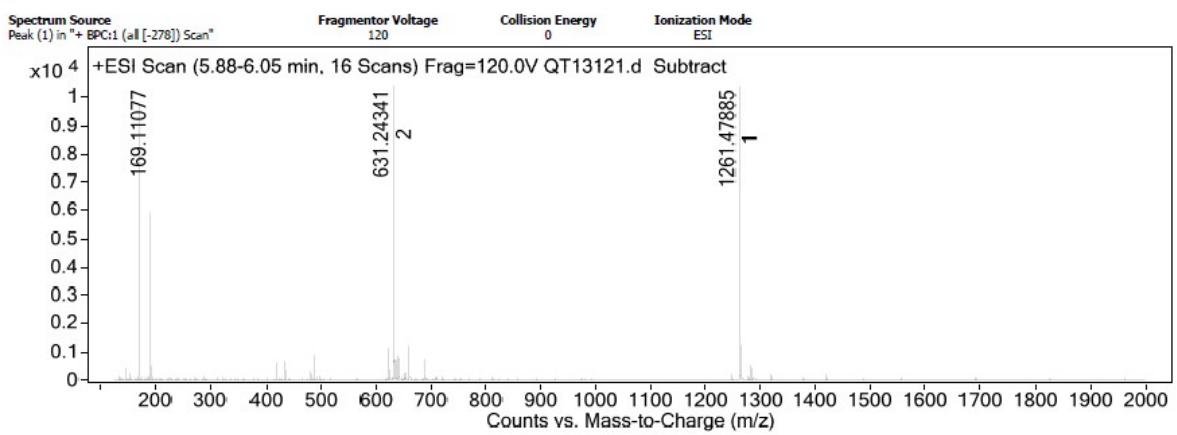

2 - 8
1
5
6
7
3
4
f1 (ppm)

HPLC chromatograms and HR Mass Analyses of FRET probes

P(5-1) Exact Mass: 1260.46987

# Peak	Retention Time	Area	% Area
1	4.089	35062	0.93
2	4.332	3736630	99.07



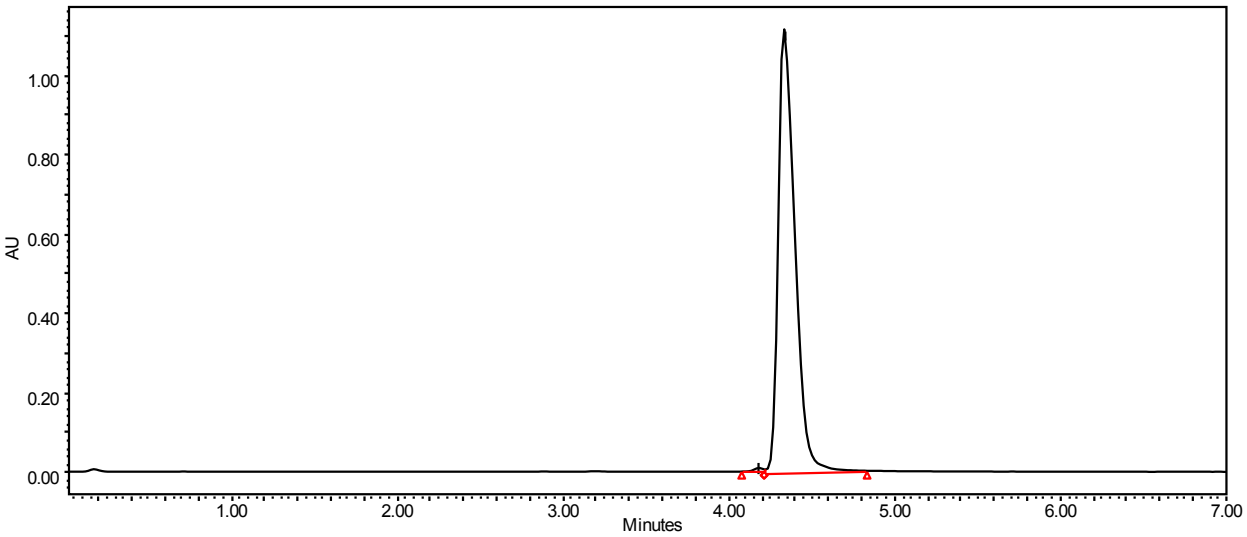


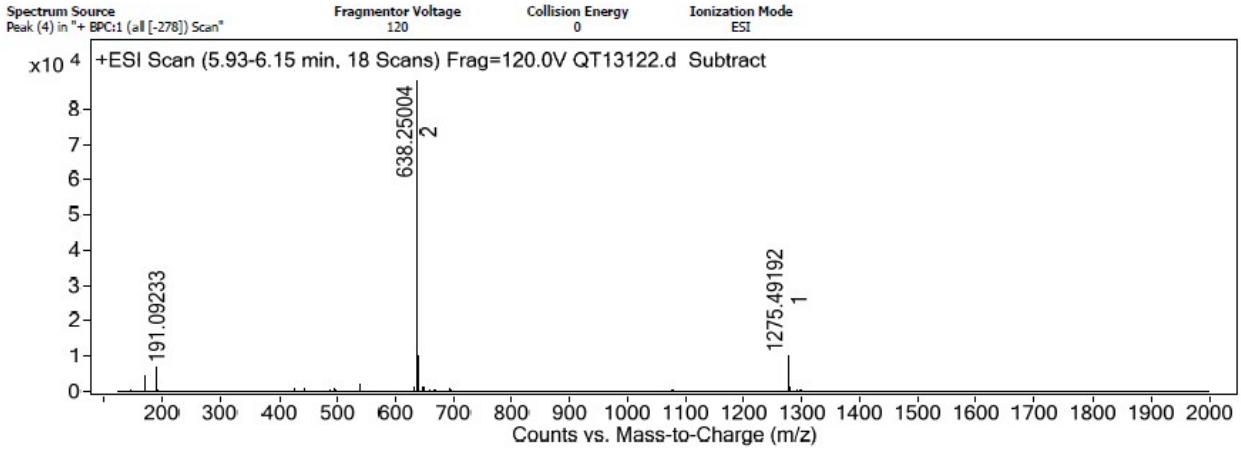
Peak List

m/z	z	Abund	Formula	Ion
1261.47885	1	10388.6	C64 H69 N12 O14 S	(M+H)+
1262.48138	1	8004.6	C64 H69 N12 O14 S	(M+H)+

P(5-2) Exact Mass: 1274.48552

# Peak	Retention Time	Area	% Area
1	4.183	35320	0.45
2	4.337	7774087	99.55





Peak List

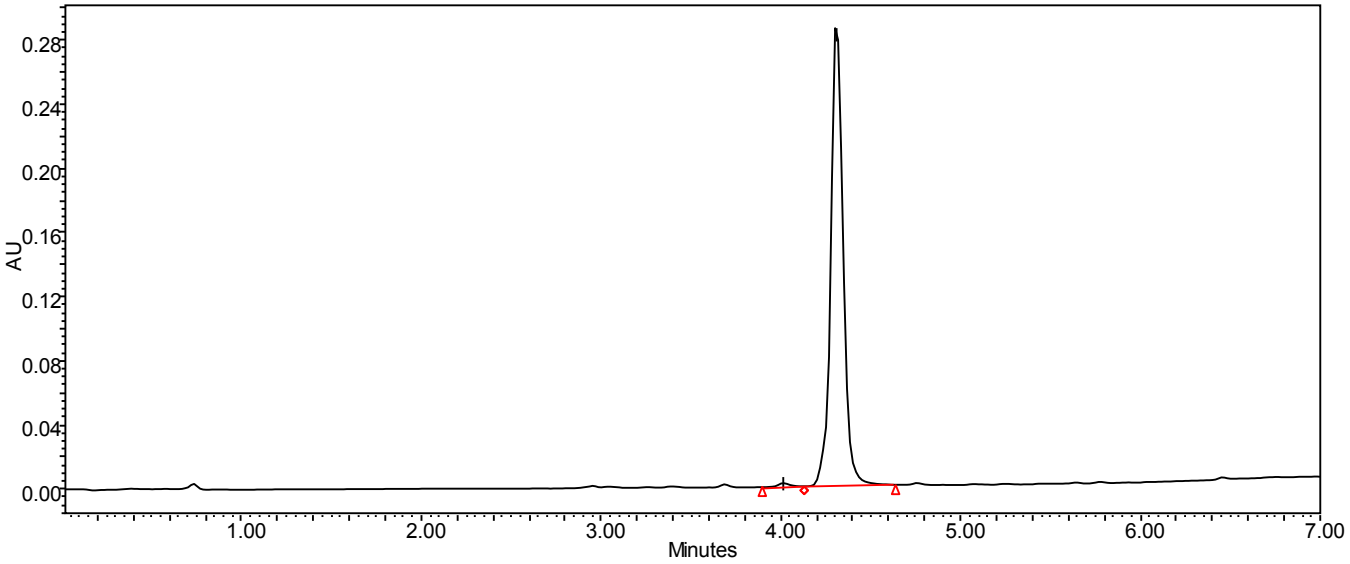
m/z	z	Abund	Formula	Ion
191.09233		6987.2		
638.25004	2	89186	C65 H72 N12 O14 S	(M+2H)+2
638.7514	2	65946.7	C65 H72 N12 O14 S	(M+2H)+2
639.25179	2	29393.8	C65 H72 N12 O14 S	(M+2H)+2
639.75182	2	10307.6	C65 H72 N12 O14 S	(M+2H)+2
1275.49192	1	9929.4	C65 H71 N12 O14 S	(M+H)+
1276.49501	1	7801.7	C65 H71 N12 O14 S	(M+H)+

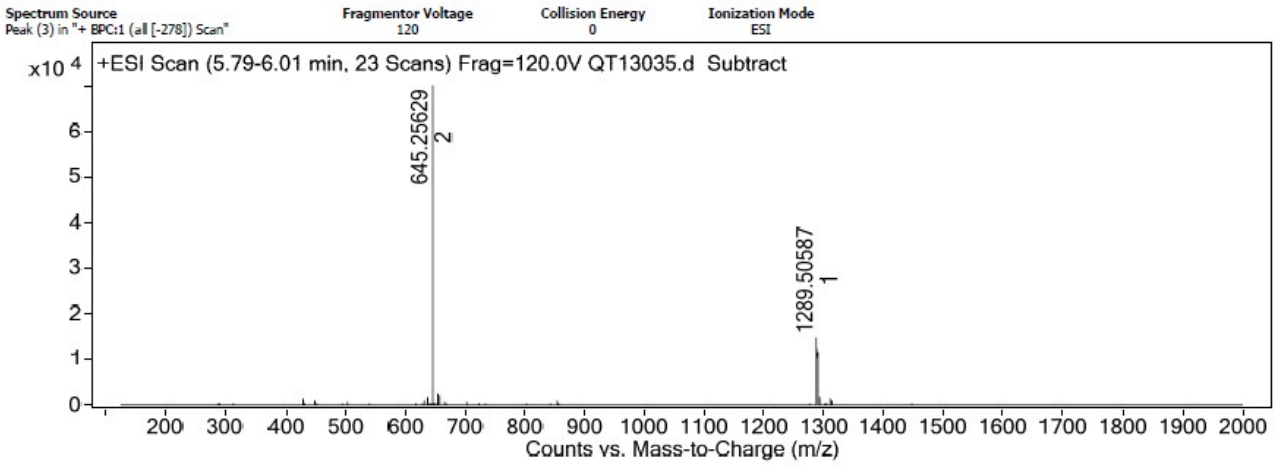
HPLC and HRMS spectra of **P(6-1)** were described in ref. 1

P(6-2)

Exact Mass: 1288.50117

# Peak	Retention Time	Area	% Area
1	4.015	9744	0.70
2	4.309	1373402	99.30





Peak List

m/z	z	Abund	Formula	Ion
645.25629	2	70377.1	C66 H74 N12 O14 S	(M+2H)+2
645.75788	2	53266.5	C66 H74 N12 O14 S	(M+2H)+2
646.2583	2	24200.7	C66 H74 N12 O14 S	(M+2H)+2
646.75833	2	8780.5	C66 H74 N12 O14 S	(M+2H)+2
1289.50587	1	14843.7	C66 H73 N12 O14 S	(M+H)+
1290.50835	1	11468	C66 H73 N12 O14 S	(M+H)+
1291.50942	1	5366.6	C66 H73 N12 O14 S	(M+H)+

References:

(1) Dovgan, I., Kolodych, S., Koniev, O., and Wagner, A. (2016) 2-(Maleimidomethyl)-1,3-Dioxanes (MD): a Serum-Stable Self-hydrolysable Hydrophilic Alternative to Classical Maleimide Conjugation. *Sci. Rep. 6*, 30835.