

Supplementary Information

Fura-2FF-based calcium indicator for protein labeling

Agostina A. Ruggiu, Michael Bannwarth and Kai Johnsson

Laboratory of Protein Engineering, Institute of Chemical Sciences and Engineering, École Polytechnique Fédérale de Lausanne (EPFL), CH-1015, Lausanne, Switzerland

kai.johnsson@epfl.ch

¹H-NMR spectra: The ¹H-NMR spectra were recorded on Bruker ARX-400 FT, Bruker DPX-400 FT, Bruker DRX-400 FT spectrometers at 400 MHz and Bruker DPX-600 FT at 600 MHz.

¹³C-NMR spectra: The ¹³C-NMR spectra were recorded on Bruker ARX-400 FT, Bruker DPX-400 FT, Bruker DRX-400 FT spectrometers at 100.6 MHz. The signals of residual solvents were used as reference.

Spectrum description:

¹H-NMR (solvent, frequency) chemical shift (δ) [ppm] (apparent multiplicity, coupling constants (*J*) [Hz], integral, assignments).

¹³C-NMR (solvent, frequency) chemical shift (δ) [ppm] (coupling constants (*J*) carbon-fluorine [Hz]).

Abbreviations: s: singlet; d: doublet; t: triplet; q: quartet; b: broad.

Mass spectrometry: The high-resolution mass spectra were recorded on a Nermag R 10-10.

Fluorescence Measurements

Fluorescence excitation and emission spectra were measured on a Molecular Devices FlexStation. Solutions of 1 μM concentration of the Fura-2FF derivatives were used, where applicable SNAP was added at 2 μM concentration or purified (NAP column) SNAP conjugates were measured. Lower calcium concentrations were adjusted with the Molecular Probes Calcium buffer kit #1 (30 mM MOPS/KOH, pH = 7.2, 100 mM KCl and between 0-10 mM EGTA or 0-10 mM CaEGTA giving the buffered free calcium value needed). Higher calcium concentrations were measured using the Molecular Probes Calcium buffer kit #3 (30 mM MOPS/KOH, pH = 7.2, 100 mM KCl and the needed amount of CaCl_2). Fluorescence intensities were measured in 96 well plates containing 200 μL of solution in each well, with either using clear-bottom micro plates (Greiner) in the bottom read mode or black micro test assay plates (Becton Dickinson) in the top read mode. The spectra were recorded at 9 nm bandwidth, which was a fixed instrument parameter. Spectra were recorded at 1 nm step-size and then averaged over 9 nm.

Ratios were calculated at excitation wavelengths of 340 nm and 380 nm with bandwidths of 20 nm: $R = F_{340\text{ nm}}/F_{380\text{ nm}}$ or at other wavelengths where indicated. Ratio quotients (RQ) or dynamic ranges, respectively, were calculated by dividing the calcium complex intensity ratio by the appropriate value of the free anion form ratio: $\text{RQ} = R_{340\text{nm}/380\text{nm, ExcessCa}^{2+}}/R_{340\text{nm}/380\text{nm, ZeroCa}^{2+}}$. Quantum yields (QY) were calculated by comparing the integrated fluorescence emission intensity of the Fura-2FF derivatives with Fura-2¹ over the range 410 – 460 nm and corrected by dividing by their respective absorbance at the excitation wavelength (360 nm):

$$QY = \frac{\text{Fluorescence}_{\text{Emitted, 410-660nm (Excitation: 360nm)} }^{\text{Fura-Derivative}}}{\text{Absorbance}_{360\text{nm}}^{\text{Fura-Derivative}}} \cdot \frac{\text{Absorbance}_{360\text{nm}}^{\text{Fura-2}}}{\text{Fluorescence}_{\text{Emitted, 410-660nm (Excitation: 360nm)} }^{\text{Fura-2}}} \cdot QY_{\text{Literature}}^{\text{Fura-2}}$$

For the uncertainty of the quantum yield the uncertainty of the original quantum yield, the one of Fura-2¹, plays a principal role. As the uncertainty of this value is not given, uncertainties of the values based on it have also not been calculated. Given the number of factors in the quantum yield calculation the absolute uncertainties are expected to be rather larger and the obtained numbers should only be used for relative comparison of the investigated compounds.

Absorbance spectra of samples containing 10 μM Fura-2FF derivatives in microcuvettes (100 μL , Hellma, filled with 120 μL or more) were recorded with a Perkin Elmer Lambda 10 UV/Vis spectrometer. Apparent calcium binding constants were calculated from the fluorescence spectra

using plots of $\log \frac{\left(R_{340\text{nm}, 380\text{nm}} - R_{340\text{nm}, 380\text{nm}, [\text{Ca}^{2+}]_{\text{free}}=0} \right) \cdot F_{\text{Ind}, \lambda_2}^C}{\left(R_{340\text{nm}, 380\text{nm}, [\text{Ca}^{2+}]_{\text{free saturating}}} - R_{340\text{nm}, 380\text{nm}} \right) \cdot F_{\text{Ca-Ind}, \lambda_2}^C}$ against $\log [\text{Ca}^{2+}]_{\text{free}}$.

Best for measurement of the calcium concentration are arguably the wavelengths where the largest signal can be seen, i.e. the maxima of the difference spectra, these maxima are stated in the second column of Table S1. Difference spectra were calculated by subtracting the excitation spectra of the calcium bound form from the free ion form.

Fura-2FF Derivative	Fluorescence difference spectra (free anion minus Ca ²⁺ bound) extrema		Ratio Quotient at the fluorescence difference spectra extrema	Quantum Yields (Fura-2 used as reference)	
	Minimum	Maximum		Zero Ca ²⁺	Excess Ca ²⁺
Fura-2FF	331 nm	375 nm	22.9	0.34	0.53
N-SNAP and Fura-2FF	330 nm	374 nm	23.1	0.34	0.46
Fura-2FF- <i>O</i> ⁶ -BG (1)	337 nm	387 nm	12.4	0.13	0.13
N-SNAP-Fura-2FF-1	337 nm	387 nm	11.7	0.26	0.26

Table S1

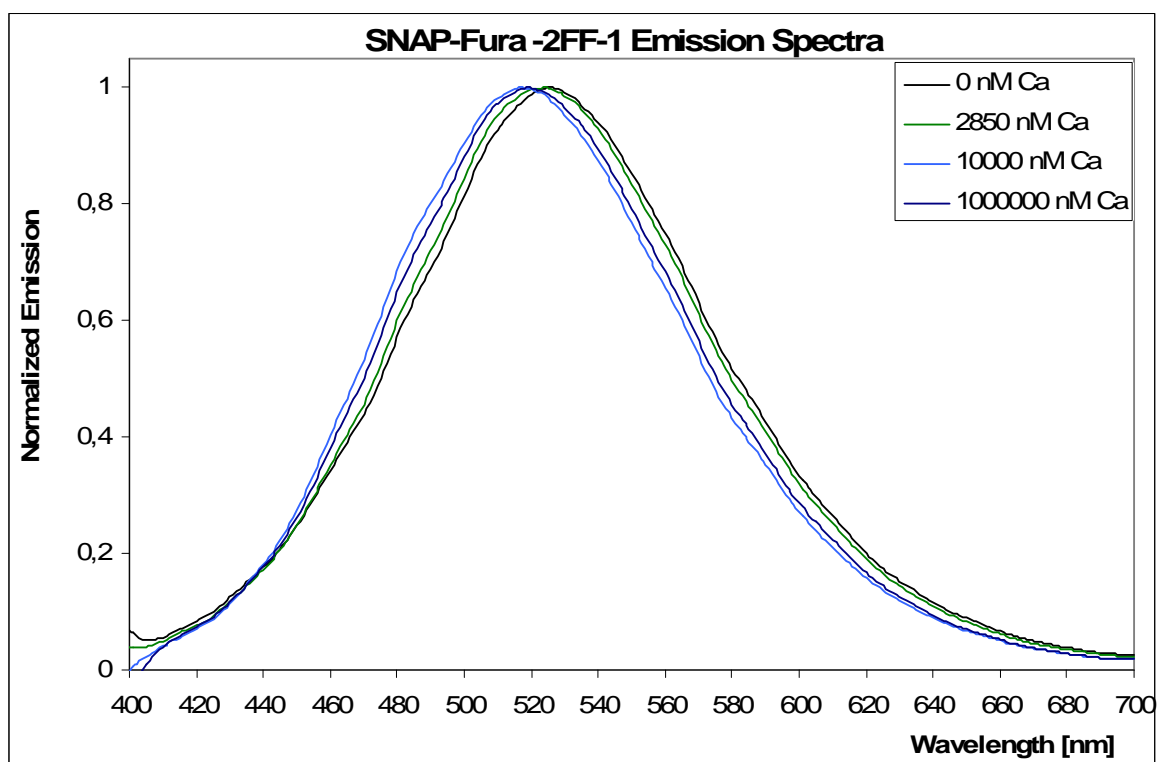
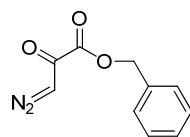


Figure S1: Fluorescence emission spectra of SNAP-Fura-2FF-1 at different calcium concentrations.

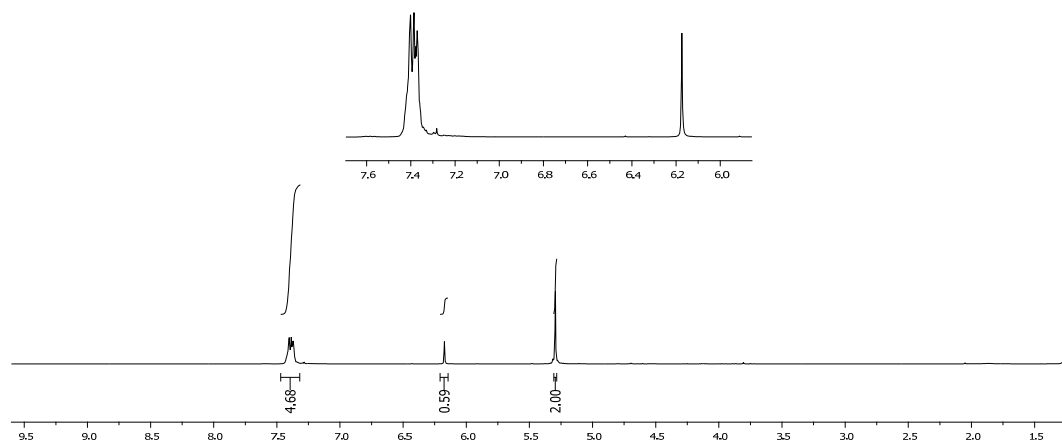
Synthesis of Benzyl 3-diazo-2-oxaproanoate (5)



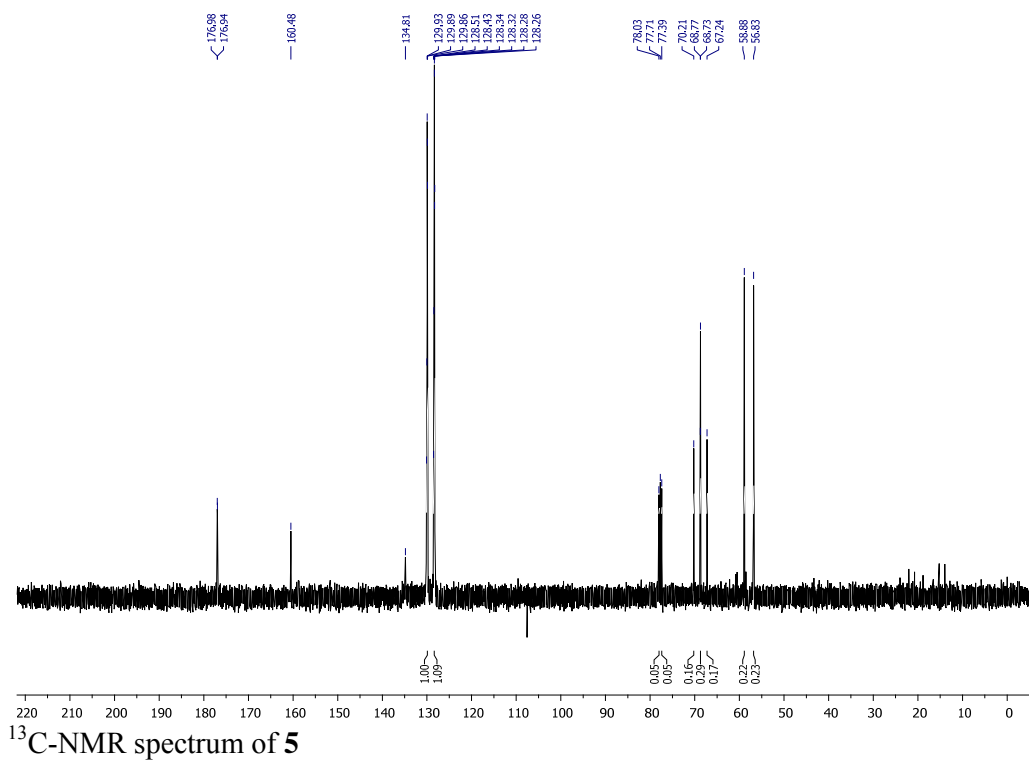
Oxalyl chloride **4** (1.98 ml, 23.6 mmol) was added to a solution of benzyl alcohol (2.44 ml, 23.6 mmol), in CH₂Cl₂ (20 mL) at 0°C. The mixture was stirred at room temperature for 1.5 h. The solvent and residual oxalyl chloride were removed. The resulting colourless oil was dissolved in THF (40 ml) and trimethylsilyldiazomethane was added carefully at 0 °C. The mixture was stirred for 12 h, then the solvent was removed and the product was purified by column chromatography on silica gel (light petroleum ether/AcOEt, 7:3) to afford the product **5** (3.87 g, 80%) as a yellow solid.

¹H-NMR (CDCl₃, 400 MHz): 7.47-7.28 (m, 5H, H-arom), 6.17 (1H, s, N₂CH), 5.29 (s, 2H, CH₂).

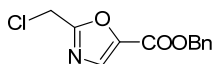
¹³C NMR (CDCl₃, 101 MHz): δ 176.98, 176.94, 160.48, 134.83, 129.23 (d, ¹J(C-H)=161, 2C), 129.10(d, ¹J(C-H) = 160, 2C), 68.73 (t, ¹J(C-H) = 149, 1C), 57.85 (d, ¹J(C-H) = 206, 1C)



¹H-NMR spectrum of **5**



Synthesis of benzyl 2-chloromethyloxazole-5-carboxylate (6)

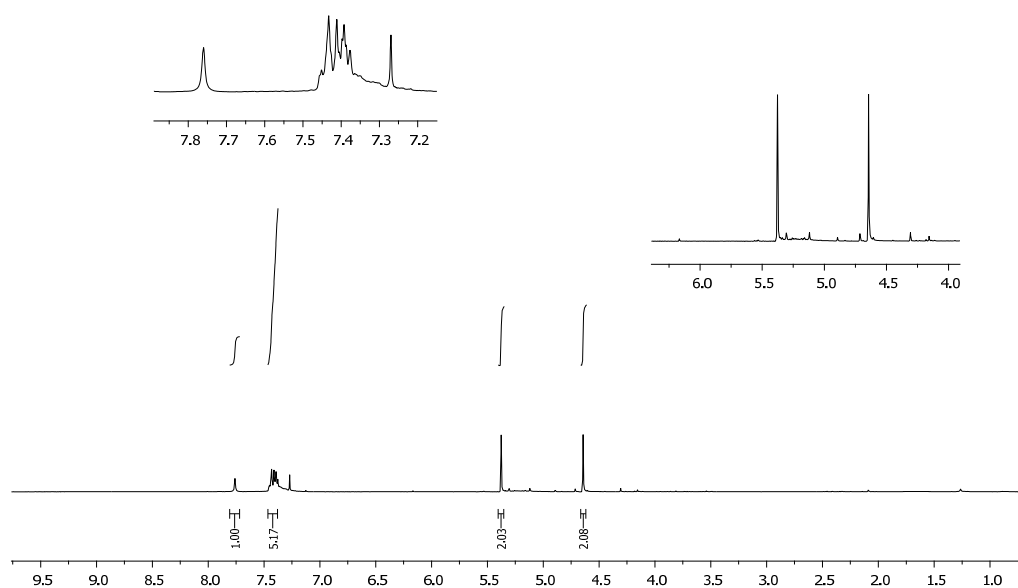


A solution of benzyldiazopyruvate **5** (3.9 g, 19 mmol), chloroacetonitrile (12 ml, 190 mmol) and few mg of Cu(acac) in toluene (20 mL) was stirred at 70 °C for 1.5. The cooled solution was partitioned with ether and NaHCO₃. The organic phases were combined, concentrated under reduced pressure, and purified by column chromatography on silica gel (petroleum ether/AcOEt, 7:3) to afford **6** (2.35 g, 50% yield) as a brown syrup.

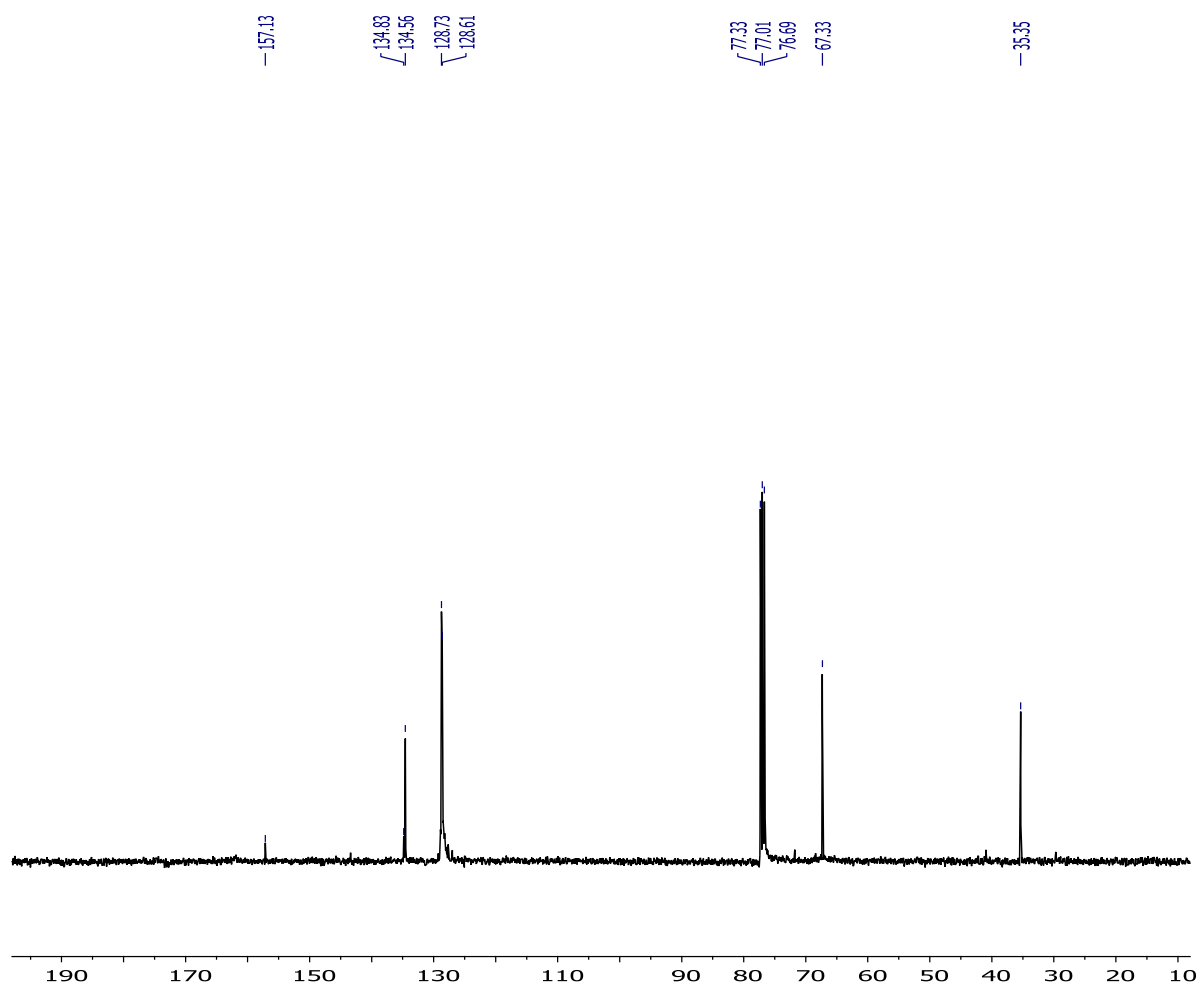
¹H-NMR (CDCl₃, 400 MHz): 7.76 (s, 1H, oxazole), 7.46-38 (m, 5H, H-arom), 5.37 (s, 2H, COOCH₂), 4.63 (s, 2H, ClCH₂).

¹³C NMR (CDCl₃, 101 MHz): 157.53, 135.25, 134.97, 129.16, 129.14 (2C), 129.12 (2C), 67.73, 35.76.

ESI-HRMS m/z, for C₁₂H₁₀ClNO₃H: Calcd. 252.0427; found 252.0421.

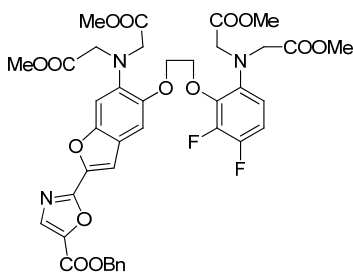


¹H-NMR spectrum of **6**



^{13}C -NMR spectrum of **6**

Synthesis of O,O',O'',O'''-tetramethyl Fura-2FF oxazole-benzyl ester (**7**)



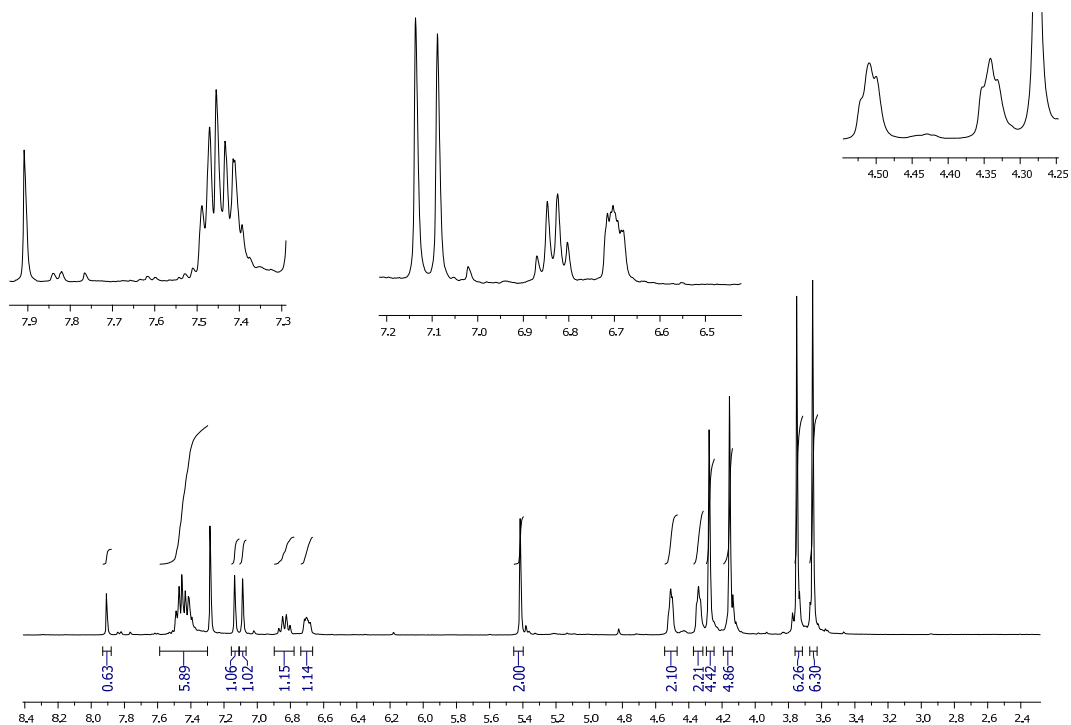
A solution of compound **3** (200 mg, 0.35 mmol), benzyl-2-chloromethyloxazole and K_2CO_3 (117 mg, 0.82 mmol) in DMF (10 mL) was stirred at 80 °C for 2 h. The cooled mixture was diluted with water, acidified with 1 M HCl and extracted with CHCl_3 . The organic layers were dried with

MgSO₄, filtered and concentrated. Purification by flash chromatography on silica gel (EtOAc/petroleum ether, 1:2 to 1:1) afforded **7** (140 mg, 50%) as a brown oil.

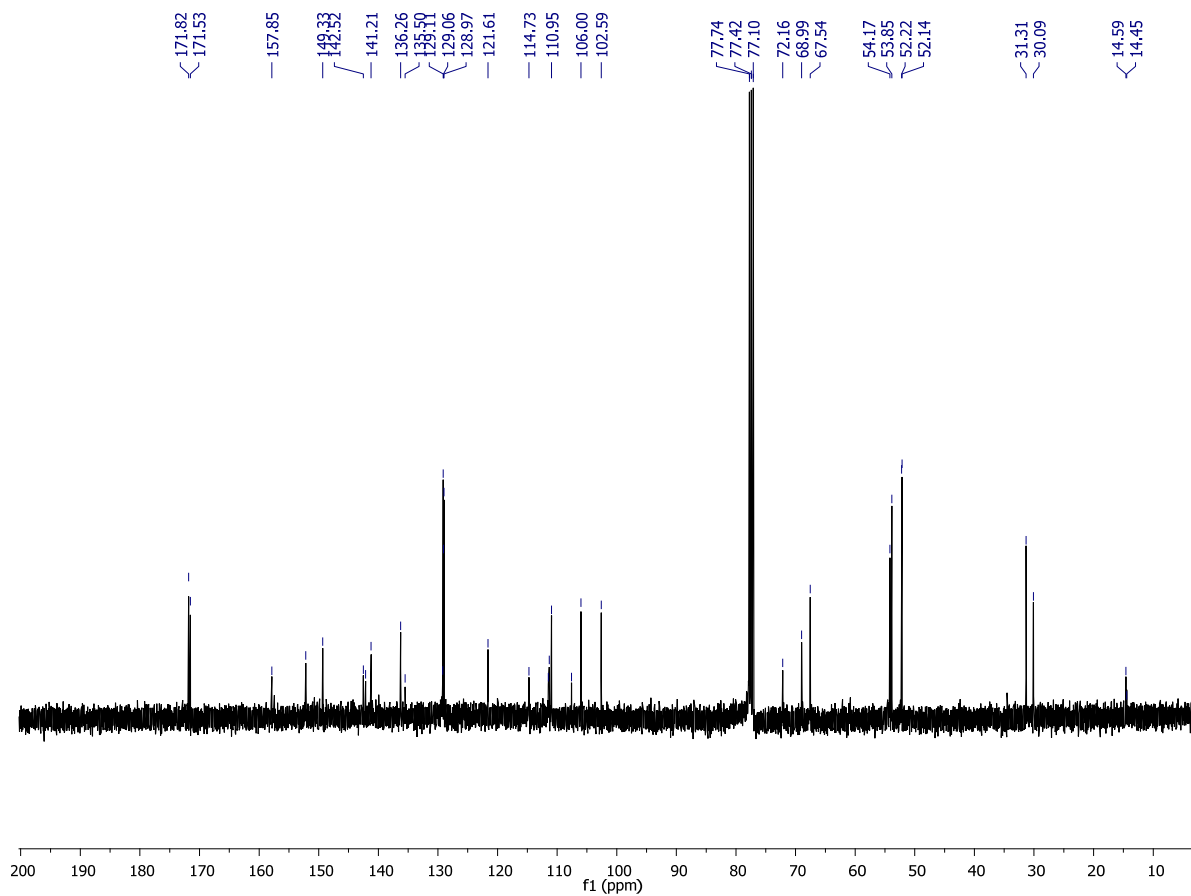
¹H-NMR (CDCl₃, 400 MHz): δ 7.90 (s, 1H, oxazole), 7.48-7.35 (m, 6H, H-arom), 1H, H-furan), 7.12 (s, 1H, H-arom), 7.07 (s, 1H, H-arom), 6.85-6.78 (m, 1H, H-arom), 6.70-6.66 (m, 1H, H-arom), 5.40 (s, 2H, CO₂CH₂Ph), 4.49 (t, 2H, ³J = 4.0, OCH₂CH₂O), 4.32 (t, 2H, OCH₂CH₂O), 4.26 (s, 4H, NCH₂COOMe), 4.14 (s, 4H, NCH₂COOMe), 3.73 (s, 6H, COOCH₃), 3.64 (s, 6H, COOCH₃).

¹³C-NMR (CDCl₃, 151 MHz): δ (171.48, 171.18, 5C), 157.43, 157.03, 151.79, 148.94, 147.13 (dd, J_{CF} = 11.5, 244.6), 145.09 (dd, J_{CF} = 14.2, 247), 142.08, 141.79, 140.77, 140.64 (dd, J_{CF} = 1.5, 9.3), 139.45, 135.71, 135.08, 128.73 (2C), 128.69, 128.59 (2C), 121.30, 114.40 (dd, J_{CF} = 3.4, 7.4), 111.00 (d, J_{CF} = 17.7), 105.53, 102.25, 71.73, 68.56, 67.19, 53.85 (2C), 53.51 (2C), 51.87(2C), 51.78 (2C).

ESI-HRMS m/z, for C₃₉H₃₇F₂N₃O₁₄Na: Calcd. 832.2141, found 832.2142

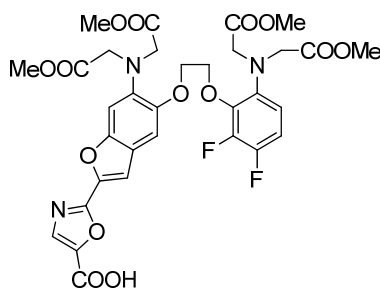


¹H-spectrum of **7**



^{13}C -NMR spectrum of **7**

Synthesis of *O,O',O'',O'''*-tetramethyl Fura-2FF (**8**)

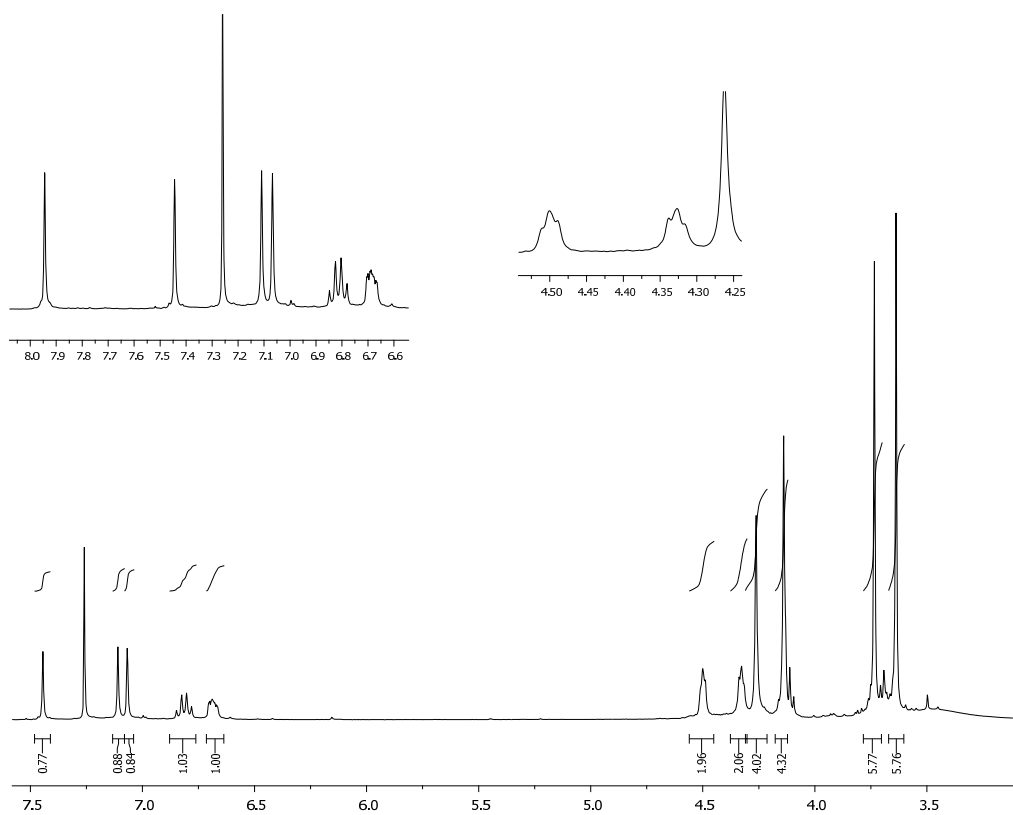


Compound **7** (20 mg, 24.9 μmol) with 10% Pd/C (5 mg) in ethyl acetate was stirred for 1 h under H_2 . After filtering through celite with ethyl acetate, the solvent was removed to give **8** as a white solid (14.5 mg, 81%).

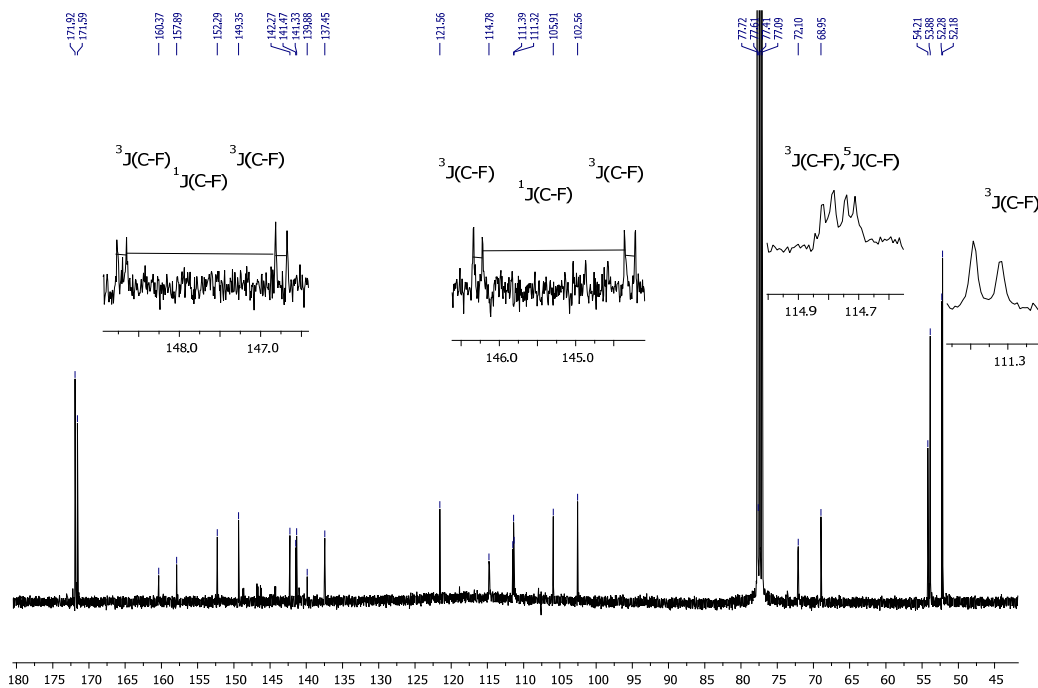
$^1\text{H-NMR}$ (CDCl_3 , 400 MHz): 7.94 (s, 1H, H-oxazole), 7.44 (s, 1H, H-furan), 7.11 (s, 1H, H-arom), 7.07 (s, 1H, H-arom), 6.85-6.78 (m, 1H, H-arom), 6.70-6.67 (m, 1H, H-arom), 4.50 (t br, $J=4.4$, 2H, $\text{OCH}_2\text{CH}_2\text{O}$), 4.33 (t, 2H, $\text{OCH}_2\text{CH}_2\text{O}$), 4.26 (s, 4H, NCH_2COOMe), 4.14 (s, 4H, NCH_2COOMe), 3.73 (s, 6H, COOCH_3), 3.64 (s, 6H, COOCH_3).

$^{13}\text{C-NMR}$ (CDCl_3 , 101 MHz): δ 171.92 (2C), 171.60 (2C), 160.37, 157.89, 152.29, 149.35, 147.73 (dd, $J_{\text{CF}} = 12.9, 197.4$), 145.28 (dd, $J_{\text{CF}} = 13.0, 200.3$), 142.27, 141.47, 141.33, 141.04 (d, $J_{\text{CF}} = 7.8$), 139.88, 137.45, 121.56, 114.76 (dd, $J_{\text{CF}} = 3.3, 7.6$), 111.49, 111.36 (d, $J_{\text{CF}} = 7.3$), 105.91, 102.56, 72.14, 68.95, 54.21 (2C), 53.88 (2C), 52.28 (2C), 52.18 (2C).

ESI-HRMS m/z , for $\text{C}_{32}\text{H}_{31}\text{F}_2\text{N}_3\text{O}_{14}\text{Na}$: Calcd. 742.1672, found 742.1705

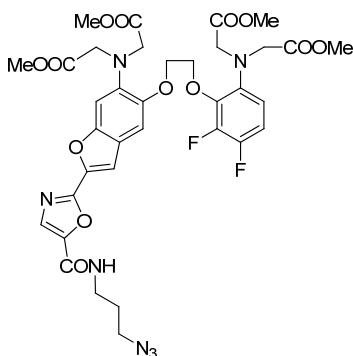


$^1\text{H-NMR}$ spectrum of **8**



$^{13}\text{C-NMR}$ spectrum of **8**

Synthesis of *O,O',O'',O'''*-tetramethyl Fura-2FF oxazole-N-(3-azidopropyl) amide (9)

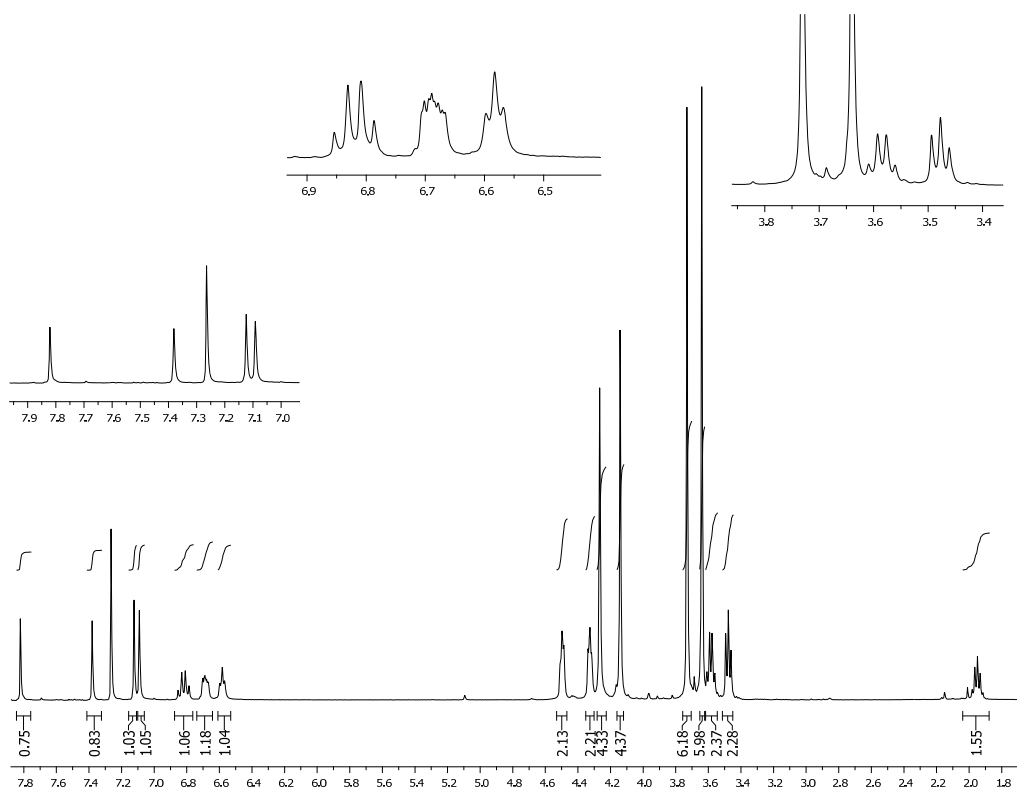


HOBt (7mg, 200 μ mol), EDAC (9.5 mg, 61 μ mol), and 3-amino-1-azido-propane (2.5mg, 25 μ mol) were added to a solution of **8** (7 mg, 9.7 μ mol) in DMF (0.5 mL). The mixture was stirred at room temperature for 1.5 h under a nitrogen atmosphere. The desired product was purified by HPLC using a linear gradient of H₂O (0.1% TFA)/CH₃CN (0.08% TFA) 99/5 to 5/95 to afford **9** (4.5 mg 59%) as a white solid.

¹H-NMR (CDCl₃, 400 MHz): δ 7.82 (s, 1H, H-arom), 7.38 (s, 1H, H-arom), 7.12 (s, 1H, H-arom), 7.09 (s, 1H, H-arom), 6.82 (dd, 1H, ³*J* = 9.0, ³*J* = 17.9, H-arom), 6.69 (dd, 1H, ³*J* = 3.9, ³*J* = 8.2, H-arom), 6.58 (t, 1H, ³*J* = 5.8, CONH), 4.50 (t br, 2H, ³*J* = 4.4, OCH₂CH₂O), 4.33 (t, 2H, OCH₂CH₂O), 4.27 (s, 4H, NCH₂COOMe), 4.14 (s, 4H, NCH₂COOMe), 3.73 (s, 6H, COOCH₃), 3.64 (s, 6H, COOCH₃), 3.59 (dd, 2H, ³*J* = 6.5, ³*J* = 12.9, NHCH₂CH₂), 3.48 (t, 2H, ³*J* = 6.5, CH₂N₃), 1.95 (m, 2H, CH₂CH₂CH₂).

¹³C-NMR (CDCl₃, 101 MHz): δ 173.95, 171.85 (2C), 171.54 (2C), 157.41, 155.40, 151.93, 149.37, 144.87, 142.64, 141.05, 139.91, 121.68, 114.72 (dd, *J*_{CF} = 3.2, 7.2), 111.42 (d, *J*_{CF} = 17.8), 110.24, 107.59, 105.98, 102.61, 77.10, 72.09, 68.99, 54.19 (2C), 53.85 (2C), 52.23 (2C), 52.17 (2C), 49.70, 37.44, 29.21.

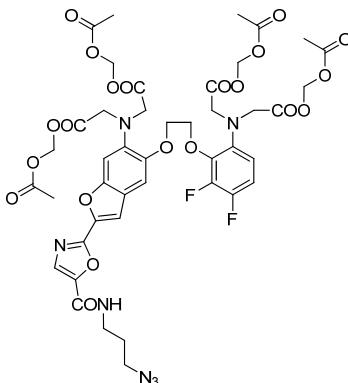
ESI-HRMS, *m/z*, for [C₃₅H₃₇F₂N₇O₁₃+H]: Calcd. 802.2495, Found 802.2491



$^1\text{H-NMR}$ spectrum of **9**

Synthesis of *O,O',O'',O'''*-tetraacetoxyl Fura-2FF oxazole-N-(3-azidopropyl) amide

(11)

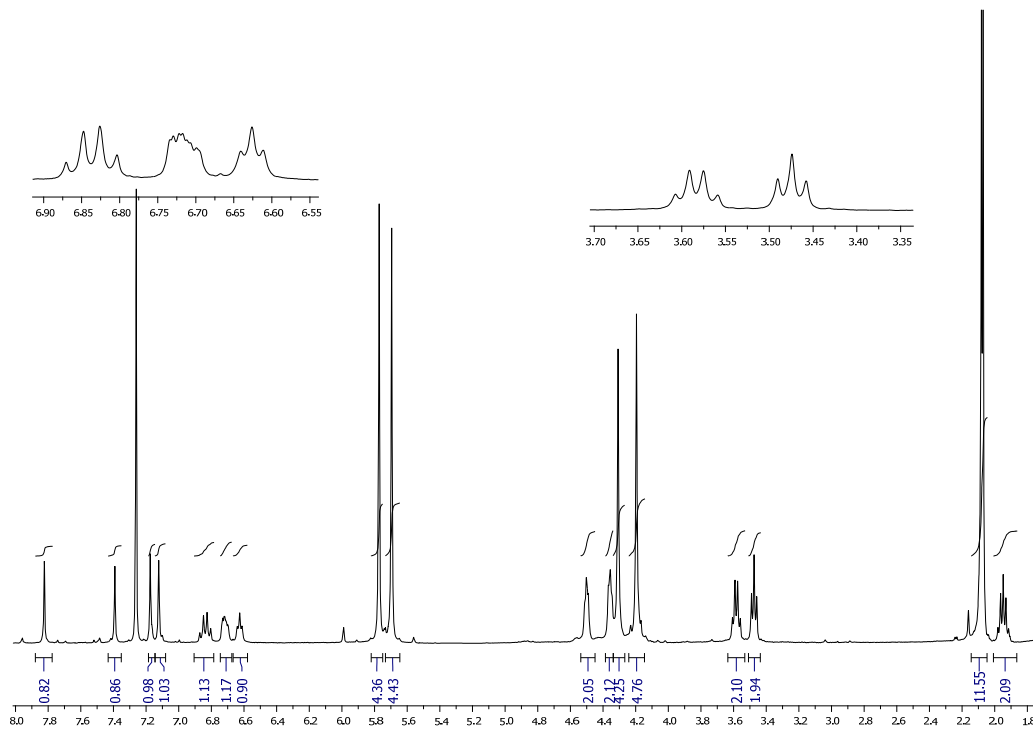


KOH (10 mg) was added to solution of compound **9** (6 mg, 7.4 μ mol) in MeOH/H₂O (1:1, 0.5 mL) and stirred overnight. The methanol was removed in vacuum and 1M HCl was added drop wise to precipitate the product. The precipitate was washed with water and dried under vacuum to obtain Fura-2FF azide tetracarboxylic acid (5.5 mg, 97%) as white solid. The tetracarboxylic acid (5 mg, 6.7 μ mol) was dissolved in DMF (0.5 mL) and DIPEA (4 μ L, 22 μ mol) and bromomethyl acetate (4.5 μ L, 44 μ mol) were added. The mixture was stirred under a nitrogen atmosphere at 0 °C for 12 h. Successively, the desired product was purified by HPLC using a linear gradient of H₂O (0.1% TFA)/CH₃CN (0.08% TFA) 99/1 to 20/80 to yield **11** (4.5 mg, 65%).

¹H-NMR (CDCl₃, 400 MHz): δ 7.82 (s, 1H, H-oxazole), 7.39 (s, 1H, H-furan), 7.17 (s, 1H, H-arom), 7.12 (s, 1H, H-arom), 6.84 (dd, ³J = 9.1, ³J = 17.6, 1H, H-arom), 6.73-6.67 (m, 1H, H-arom), 6.63 (t, 1H, ³J = 5.9, CONH), 5.77 (s, 4H COOCH₂COOMe), 5.69 (s, 4H COOCH₂COOMe), 4.50 (t br, 2H, OCH₂CH₂O), 4.36 (t br, 2H, OCH₂CH₂O), 4.31 (s, 4H, NCH₂COOMe), 4.19 (s, 4H, NCH₂COOMe), 3.58 (dd, 2H ³J = 6.5, ³J = 12.8, CONHCH₂), 3.47 (t, 2H, ³J = 6.5, CH₂N₃), 2.08, 2.07 (s, 12H, COOCH₂COOCH₃), 1.95 (m, 2H, CH₂CH₂CH₂).

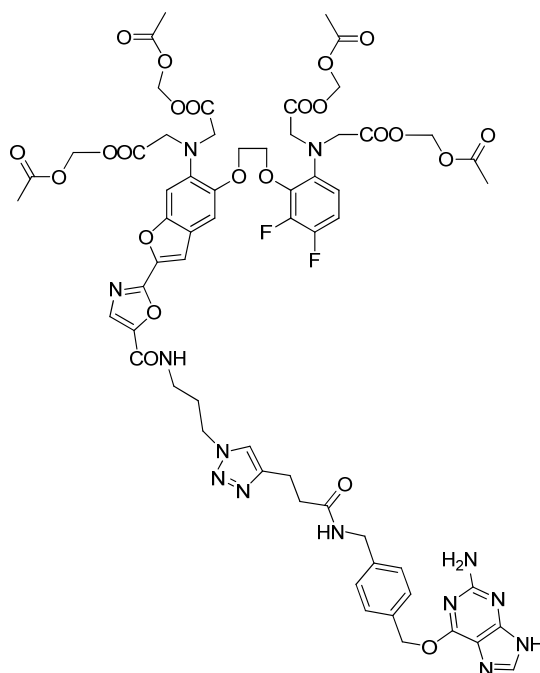
¹³C-NMR (CDCl₃, 151 MHz): δ 169.68, 169.53, 169.50, 169.36, 9C), 156.99, 154.85, 151.42, 149.14, 147.36 (dd, *J*_{CF} = 11.9, 245.9), 145.25 (dd, *J*_{CF} = 13.8, 247.6), 144.61, 142.61, 140.89 (d, *J*_{CF} = 11.0), 140.01, 138.93, 121.89, 114.83 (dd, *J*_{CF} = 3.2, 7.5), 111.22 (d, *J*_{CF} = 17.8), 109.72, 106.26, 103.12, 79.38 (2C), 79.24 (2C), 71.79, 68.83, 53.74(2C), 53.44(2C), 49.29, 37.04, 28.84, 20.63 (2C), 20.62 (2C).

ESI-HRMS *m/z*, for C₄₃H₄₅F₂N₇O₂₁Na: Calcd. 1056.2534, found 1056.2587.



$^1\text{H-NMR}$ spectrum of **11**

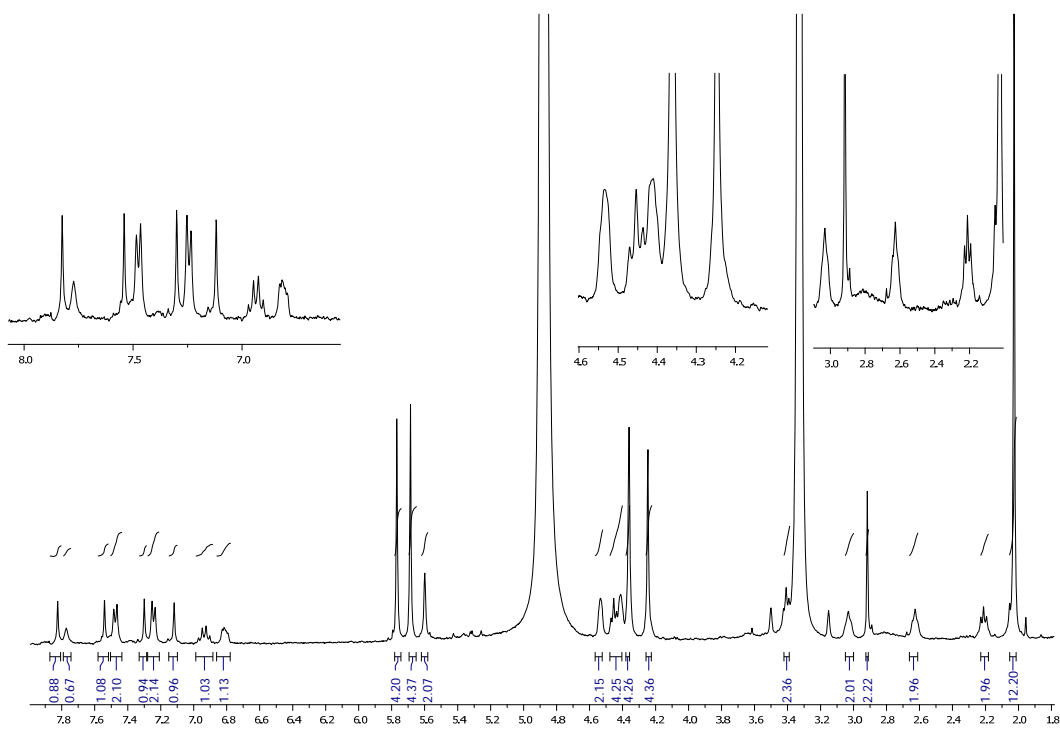
Synthesis of *O,O',O'',O'''*-tetraacetoxy Fura-2FF-*O*⁶-Benzylguanine (2)



CuSO₄·5H₂O (4 μL, 0.6 μmol, from a stock of 25 mg/mL in water), sodium ascorbate (4.5 μL, 1.3 μmol, from a stock of 25 mg/mL in water) and BG derivative **10** (obtained from Covalys Biosciences) were added to a solution of **11** (3.6 mg, 6.7 μmol) in CH₂Cl₂/iPrOH/H₂O (2:1:2, 500 μL). The mixture was stirred at room temperature for 4 days. Successively, the mixture was purified by HPLC using a linear gradient of H₂O (0.1% TFA)/CH₃CN (0.08% TFA) 95/5 to 5/95 to afford **2** (1.5 mg, 35%).

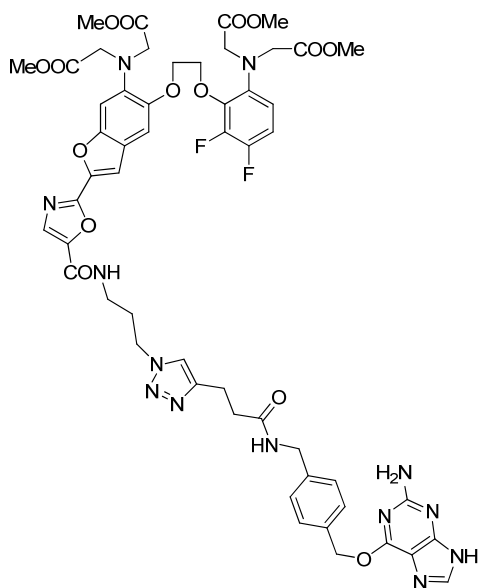
¹H-NMR (MeOD, 400 MHz): δ 7.82 (s, 1H, H-arom), 7.77 (s, 1H, H-arom), 7.54 (s, 1H, H-arom), 7.47 (d, 2H, ³J = 7.8, H-arom), 7.30 (s, 1H, H-arom), 7.24 (d, 2H, ³J = 7.8, H-arom), 7.12 (s, 1H, H-arom), 6.94 (d, 1H, ³J = 8.8, H-arom), 6.82 (s, 1H, H-arom), 5.77 (s, 4H, COOCH₂COOMe), 5.69 (s, 4H, COOCH₂COO), 5.60 (s, 2H, ArCH₂BG), 4.53 (s, 2H, OCH₂CH₂O), 4.48 – 4.40 (m, 4H, CONHCH₂CH₂CH₂, OCH₂CH₂O), 4.36 (s, 4H, NCH₂COO), 4.25 (s, 4H, NCH₂COO), 3.40 (t br, 2H, CONHCH₂CH₂CH₂), 3.03 (s, 2H, CCH₂CH₂CONH), 2.92 (s, 2H, CONHCH₂Ar), 2.63 (s, 2H, CCH₂CH₂CONH), 2.26 – 2.18 (m, 2H, CONHCH₂CH₂CH₂), 2.04 (s, 12H, CH₂COOCH₃).

ESI-HRMS m/z, for [C₆₁H₆₃F₂N₁₃O₂₃/2]: Calcd. 692.2183, found 692.6525.



¹H-NMR spectrum of **2**

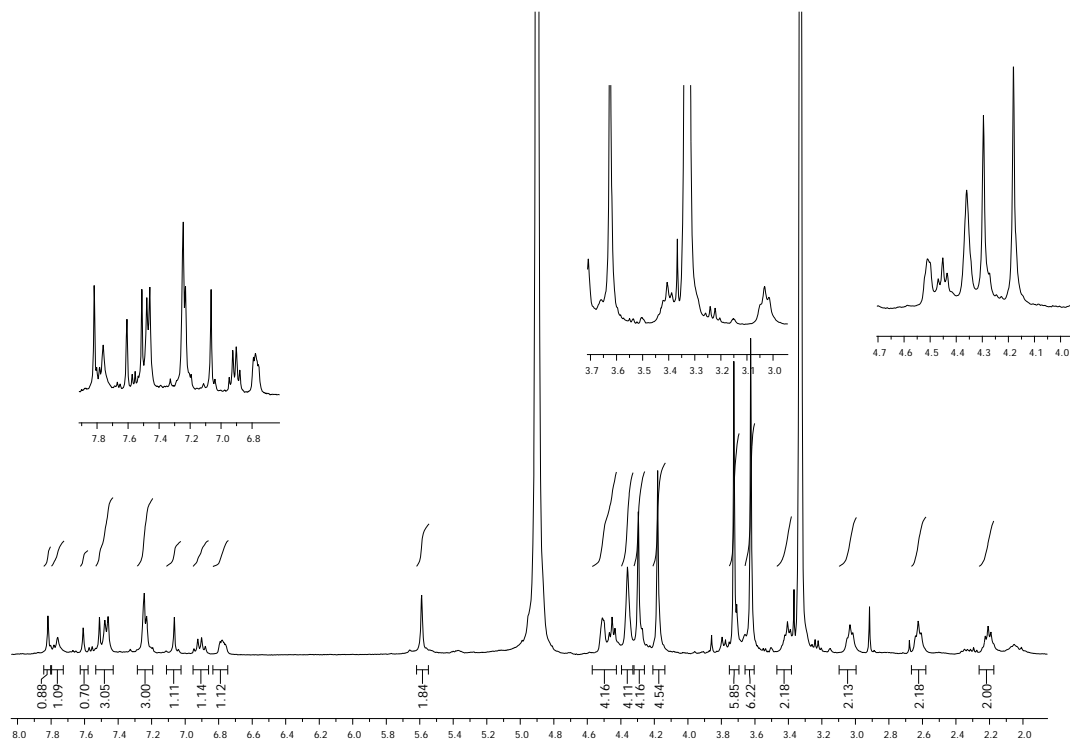
Synthesis of *O,O',O'',O'''*-tetraacetoxy Fura-2FF-O6-Benzylguanine (12)



CuSO₄·5H₂O (4 μL, 0.6 μmol, from a stock of 25mg/mL in water), sodium ascarbate (4.5 μL, 1.3 μmol, from a stock of 25mg/mL in water) and product BG derivative **10** were added to a solution of **9** (5.5 mg, 6.8 μmol) in CH₂Cl₂/iPrOH/H₂O (2:1:2, 500 μL). The mixture was stirred at room temperature for 1 day. Successively the mixture was purified by HPLC using a linear gradient of H₂O (0.1% TFA)/CH₃CN (0.08% TFA) 95/5 to 5/95 to afford **13** (4.8 mg, 61%).

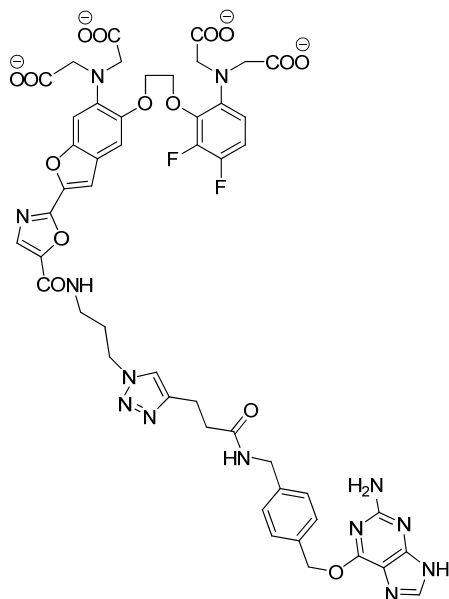
H-NMR (CDCl₃, 400 MHz): δ 7.84 – 7.73 (m, 2H, H-arom), 7.61 (s, 1H, H-arom), 7.53 – 7.43 (m, 3H, H-arom), 7.24 (m, 3H, H-arom), 7.06 (s, 1H, H-arom), 6.91 (dd, 1H, *J* = 9.3, 18.1, H-arom), 6.78 (s, 1H, H-arom), 5.59 (s, 2H, ArCH₂BG), 4.47 (m, 4H, 2H CONHCH₂CH₂CH₂, 2H, OCH₂CH₂O), 4.36 (s, 4H, OCH₂CH₂O, CONHCH₂Ar), 4.30 (s, 4H, NCH₂COO), 4.18 (s, 4H, NCH₂COO), 3.72 (s, 6H, NCH₂COOMe), 3.63 (s, 6H, NCH₂COOMe), 3.40 (d, 2H, ³*J* = 6.7, CONHCH₂CH₂CH₂), 3.03 (s, 2H, CCH₂CH₂CONH), 2.63 (s, 2H, CCH₂CH₂CONH), 2.29 – 2.16 (m, 2H, CONHCH₂CH₂CH₂).

ESI-HRMS *m/z* for [C₅₃H₅₅F₂N₁₃O₁₅ +H]: Calcd. for 1152.3988, found 1152.3982.



$^1\text{H-NMR}$ spectrum of **12**

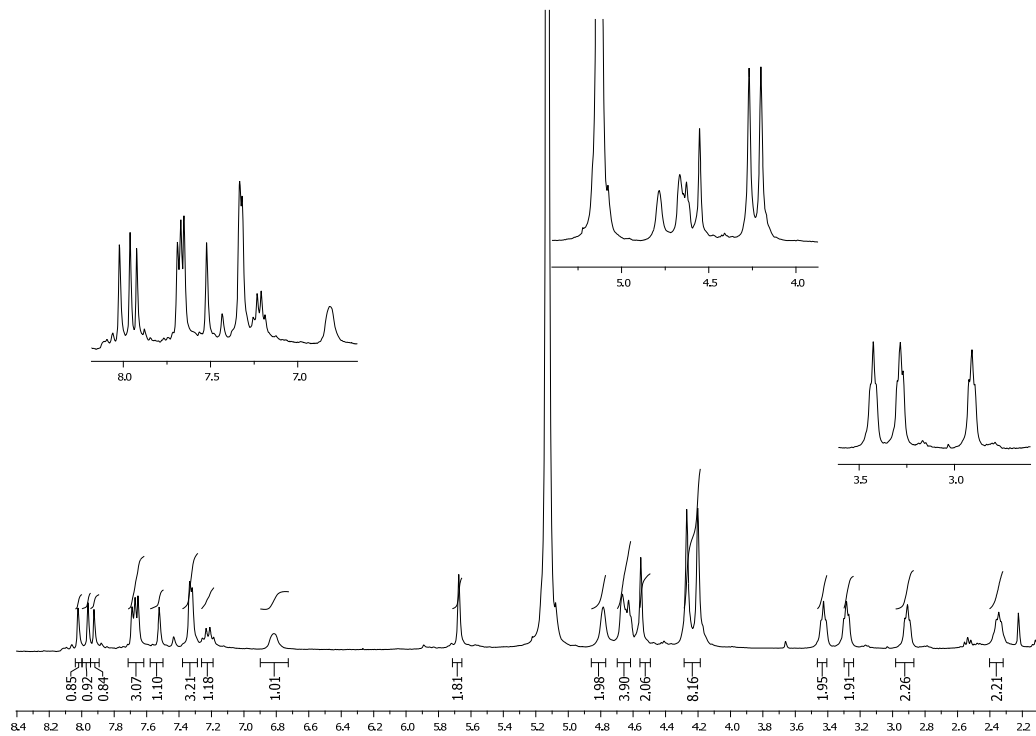
Synthesis of Fura-2FF-O⁶-Benzylguanine (1)



To solution of compound **12** (5 mg) in MeOH/H₂O (0.5 mL) was added KOH (5 mg) and stirred overnight. The solvent was removed in vacuum.

¹H NMR (400 MHz, D₂O): δ 8.04 – 7.91 (m, 2H, H-arom), 7.71 – 7.62 (m, 3H, H-arom), 7.52 (s, 1H, H-arom), 7.33 (d, *J* = 5.7, 3H, H-arom), 7.22 (d, *J* = 9.2, 2H, H-arom), 6.82 (s, 1H, H-arom), 5.67 (s, 2H, ArCH₂BG), 4.78 (s, 2H, OCH₂CH₂O), 4.65 (s, 4H, CONHCH₂CH₂CH₂, OCH₂CH₂O), 4.55 (s, 2H, CONHCH₂Ar), 4.24 (s, 8H, NCH₂COO), 3.43 (s, 2H, CONHCH₂CH₂CH₂) 3.28 (s, 2H, CCH₂CH₂CONH), 2.91 (s, 2H, CCH₂CH₂CONH), 2.34 (s, 2H, CONHCH₂CH₂CH₂).

ES-HRMS *m/z* for [C₅₃H₅₅F₂N₁₃O₁₅+5H]: Calcd. 1096.33828, found 1096.33595



$^1\text{H-NMR}$ spectrum of **1**

SNAP-tag and SNAP-Fura-2FF-1

The SNAP-tag variant used in this work was described previously², and is a truncated version of human wild-type O⁶-alkylguanine-DNA alkyltransferase (AGT) (182 amino acids) that carries the following mutations: K32I, L33F, C62A, Q115S, Q116H, K125A, A127T, R128A, G131K, G132T, M134L, R135S, C150Q, S151G, S152D, G153L, A154D, N157G, and S159E. SNAP-tag was expressed in Escherichia coli BL21 (DE3) with the expression plasmid pET-431.b as NusA fusion with a hexahistidine tag and purified by metal chelate chromatography. The NusA fusion was subsequently cleaved by thrombin and purified by ion exchange chromatography (MonoQ).

The following cloning primers for SNAP-tag were used:

MB8_NusPshAIAGTsmc_forw
GACGACAAGAGTCCGATggataaagactgcgaaatg

MB11_BamHIAGTsmc_reve
EndGlyLeuGlyProLysGlyLeuArg
attggatccTTAgcccaggcctggtttaccag

The resulting peptide preceding SNAP-tag after thrombin cleavage was (final M is the starting methionine of SNAP-tag): GSAGSGTIDDDDNDKSPM...

The velocity of the reaction of the Fura-2FF BG derivative with SNAP-tag were determined in a competition assay with BGFL. SNAP-tag was incubated with BGFL and varying concentrations of the BG-Fura-2FF derivative in PBS. The amount of SNAP-tag that reacted with BGFL was determined by fluorescence scanning after SDS-PAGE (Pharos FX Molecular Imager, Bio-Rad). SNAP-tag reacted with Fura-2FF-O⁶-benzylguanine (**1**) both in the presence of EGTA and in the presence of calcium (0.5 mM). Figure S2 shows an SDS gel of SNAP-Fura-2FF-1 followed by detection through UV illumination of the gel and recording of the fluorescence of Fura-2FF.

Ca²⁺	-	+	-	+	-	+
Fraction	T	T	S	S	P	P

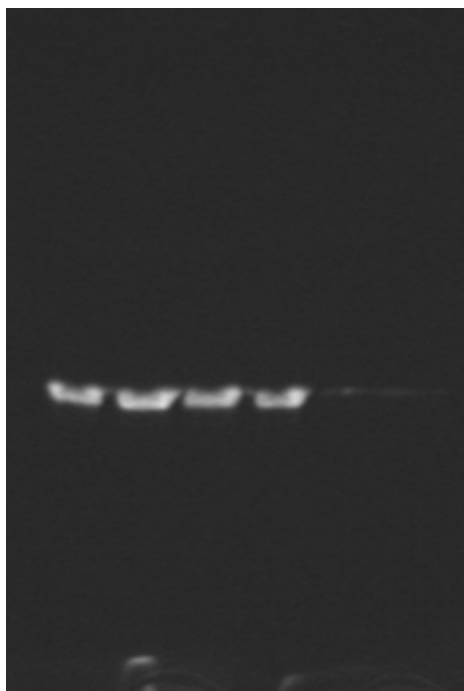


Figure S2: SDS-PAGE of SNAP-Fura-2FF-1 and analysis through UV illumination of the gel and recording of the fluorescence of Fura-2FF. The presence of EGTA and calcium (0.5 mM) is shown by minus (-) and plus (+) signs, respectively. The shown labeling was performed with crude fractions obtained during a purification of SNAP-tag from *E. coli*, demonstrating a covalent linkage of the dye to the protein and the specificity of the labeling as no other proteins are labeled. The fractions are: total (T), supernatant after centrifugation (S) and precipitate after centrifugation (10 min / 13.000 *g) (P).

1. Grynkiewicz, G.; Poenie, M.; Tsien, R. Y., A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *The Journal of biological chemistry* **1985**, 260, (6), 3440-50.
2. Gronemeyer, T.; Chidley, C.; Juillerat, A.; Heinis, C.; Johnsson, K., Directed evolution of O⁶-alkylguanine-DNA alkyltransferase for applications in protein labeling. *Protein Eng Des Sel* **2006**, 19, (7), 309-16.