Functionalizable Red Emitting Calcium Sensor Bearing a 1,4-triazole Chelating Moiety

Supplementary Information

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1. Materials and general methods

All the solvents were of analytical grade. Chemicals were purchased from commercial sources. The salts used in stock solutions of metal ions were CaCl₂, 2H₂O, CdCl₂, CuCl₂·2H₂O, FeCl₃·6H₂O, HgCl₂, KCl, MgCl₂·6H₂O, MnCl₂·4H₂O, NaCl, Zn(NO₃)₂. ¹H-NMR and ¹³C-NMR were measured on a Bruker avance III-300 MHz spectrometer with chemical shifts reported in ppm (TMS as internal standard). Mass spectra were measured on a Focus GC / DSQ II spectrometer (ThermoScientific) for IC and an API 3000 spectrometer (Applied Biosystems, PE Sclex) for ES. All pH measurements were made with a Mettler Toledo pH-Meter. Fluorescence spectra were recorded on a JASCO FP-8300 spectrophotometer. Absorption spectra were determined on a VARIAN CARY 300 Bio UV-Visible spectrophotometer. All measurements were done at a set temperature of 25°C. The purity of the dyes were checked by RP-HPLC C-18, eluant: ACN 0.1% TFA/Water 0.1% TFA, method: 20/80 to 100/0 within 20 min then 100/0 for 10 min. detection at λ₅₂₀ = 254 nm.

Synthesis

1 was synthesized according to a published protocol¹

\[
\begin{align*}
\text{To a cooled (0°C) solution of } & \text{1 (3.200 g, 15.31 mmol) in DMF (30 mL) was added propargyl bromide 80 wt. % in toluene (2.52 mL, 22.96 mmol, 1.5 eq) and K₂CO₃ (3.168 g, 22.96 mmol, 1.5 eq). The solution was heated at 80°C for 5h before being cooled down to room temperature. The solvents were evaporated and the product was extracted with EtOAc washed with water (3 times) and brine (2 times). The organic phase was dried over MgSO₄, filtered and evaporated. The crude was purified by column chromatography on silica gel (Cyclohexane/EtOAc : 9/1) to obtain the 3.28 g of } & \text{2 (86%) as a yellowish syrup. Rf=0.69 (Cyclohexane/EtOAc, 8/2).} \\
\end{align*}
\]

¹H-NMR (300 MHz, CDCl₃): δ 8.13 (d, J = 5.3 Hz, 1H, HAr), 7.10 (s, 1H, NH), 7.02-7.00 (m, 3H, H Ar), 4.78 (d, J = 2.4 Hz, 2H, CH₂), 2.58 (t, J = 2.4 Hz, 1H, CH), 1.56 (s, 9H, tBu). ¹³C-NMR (75 MHz, CDCl₃): δ 152.72 (CO Boc), 145.54 (Cq Ar), 128.64 (Cq Ar), 122.21 (CH Ar), 122.15 (CH Ar), 118.53 (CH Ar), 111.74 (CH Ar), 80.42 (Cq tBu), 78.20 (C≡CH), 76.07 (C≡CH), 56.47 (CH₃), 28.40 (tBu). MS (Cl), calcd for C₁₉H₁₇NO₃ [M]+ 247.1, found 247.1, HRMS (Cl), C₁₉H₁₇NO₃ [M]+ 247.1208, found 247.1195.

\[
\begin{align*}
\text{To a cooled (0°C) solution of } & \text{2 (3.280 g, 13.28 mmol) in DCM (20 mL) was added TFA (5 mL). The solution was allowed to stir at room temperature overnight. The TFA was neutralized by addition of a saturated solution of NaHCO₃ to reach a pH of 8. The product was extracted with DCM and the solution was dried over MgSO₄, filtered and evaporated to obtain 3. Rf=0.35 (Cyclohexane/EtOAc, 8/2).} \\
\end{align*}
\]

To a solution of 3 (1.923 g, 13.06 mmol) in acetonitrile (26 mL) were added methyl bromoacetate (3.69 mL, 39.84 mmol, 3 eq) and DIEA (6.92 mL, 39.84 mmol, 3 eq) before being warmed up to 90°C.

overnight. 2 more equivalent of both methyl bromoacetate and DIEA were then added to complete the reaction. The solution was stirred at 90°C over 6h. The solvents were evaporated, the product was extracted with DCM and washed with water. The organic phase was dried over MgSO₄, filtered and evaporated. The crude was purified by column chromatography on silica gel (Cyclohexane/EtOAc: 9/1) to obtain the 3.64 g (94%) as a yellowish syrup. Rf=0.28 (Cyclohexane/EtOAc, 8/2).

**1H-NMR** (300 MHz, CDCl₃): δ 6.95 (m, 4H, H Ar), 4.72 (d, J = 2.4 Hz, 2H, OCH₂), 4.18 (s, 4H, NCH₂), 3.75 (s, 6H, OMe), 2.52 (t, J = 2.4 Hz, 1H, CH).

**13C-NMR** (75 MHz, CDCl₃): δ 171.83 (CO esters), 149.38 (C Ar), 139.73 (C Ar), 129.95 (C Ar), 127.12 (CH Ar), 119.56 (CH Ar), 115.37 (CH Ar), 78.80 (C=CH), 56.81 (OCH₂), 53.77 (NCH₂), 51.82 (OME). MS (ES⁺), calcd for C₁₅H₁₃NO₅Na [M + Na⁺] 314.1, found 314.4. HRMS (ES⁺), calcd for C₁₅H₁₃NO₅ [M + H⁺] 292.1179, found 292.1197.

To a solution of 4 (3.06 g, 10.51 mmol) in DMF (10 mL) was slowly added POCl₃ (7.82 mL, 84.08 mmol, 8 eq). The mixture turned black and was allowed to stir at 80°C for 3 hours before being cooled down to room temperature. The mixture was then poured in water (1L) and the product was extracted with EtOAc (3 times) and washed with brine twice. The organic phase was dried over MgSO₄, filtered and evaporated to 50 mL EtOAc. The product precipitated under cooling and was filtered to obtain 2.262 g of 5 (67%) as a brown powder. **1H-NMR** (300 MHz, CDCl₃): δ 9.83 (s, 1H, CHO), 7.48-7.43 (m, 2H, H Ar), 6.80 (d, J = 8.2 Hz, 1H, H Ar), 4.74 (d, J = 2.2 Hz, 2H, OCH₂), 4.25 (s, 4H, NCH₂), 3.81 (s, 6H, OMe), 2.56 (t, J = 2.1 Hz, 1H, CH), 171.18 (CO esters), 149.33 (C Ar), 145.36 (C Ar), 129.95 (C Ar), 127.12 (CH Ar), 116.91 (CH Ar), 112.81 (CH Ar), 77.72 (C=CH), 76.10 (C=CH), 56.71 (OCH₂), 54.08 (NCH₂), 52.16 (OME). MS (Cl), calcd for C₁₆H₁₈NO₅ [M+H⁺] 320.1, found 320.1, HRMS (Cl), C₁₆H₁₈NO₅ [M+H⁺] 320.1129, found 320.1191.

To a solution of 5 (500 mg, 1.567 mmol) and methyl 2-azido acetate (360 mg, 3.134 mmol, 2 eq) in dioxane (16 mL) was added an heterogeneous solution of CuSO₄·5H₂O (195 mg, 0.783 mmol, 0.5 eq) and sodium ascorbate (217 mg, 1.097 mmol, 0.7 eq) in water (1 mL). The mixture was stirred at 50°C overnight before being extracted with DCM and washed successively with water and brine. The organic phase was dried over MgSO₄, evaporated and the crude was purified by column chromatography on silica gel (EtOAc) to obtain 571 mg of 8 (83%) as a yellowish syrup. Rf=0.40 (100% EtOAc). **1H-NMR** (300 MHz, CDCl₃): δ 9.72 (s, 1H, CHO), 7.75 (s, 1H, H triazol), 7.42 (d, J = 1.8 Hz, 1H, H Ar), 7.33 (dd, J = 8.2, 1.8 Hz, 1H, H Ar), 6.71 (d, J = 8.2 Hz, 1H, H Ar), 5.18 (s, 2H, CH₂COOME), 4.02 (t, J = 4.2 Hz, 4H, NCH₂), 3.74 (s, 3H, OMe), 3.60 (s, 6H, OMe). **13C-NMR** (75 MHz, CDCl₃): δ 190.52 (CHO), 171.15 (CO esters), 166.53(COOME), 149.08 (C Ar), 145.37 (C Ar), 143.31 (C Ar), 130.11 (CH Ar), 126.62 (CH triazol), 124.92 (CH Ar), 117.04 (CH Ar), 112.79 (CH Ar), 62.52 (CH₂COOME), 53.90 (NCH₂), 53.13 (OME), 52.00 (2 OMe), 50.77 (OCH₂). MS (Cl), calcd for C₁₅H₁₃N₂O₃ [M + H⁺] 345.1, found 345.0. HRMS (ES⁺), calcd for C₁₅H₁₃N₂O₃ [M + H⁺] 345.1510, found 345.1516.

To a solution of 8 (409 mg, 1.282 mmol) and methyl 2-azido acetate (294 mg, 2.564 mmol, 2 eq) in dioxane (15 mL) was added Cp*RuCl(PPh₃)₂ (40 mg, 0.05 mmol, 0.04 eq). The solution was allowed to stir at 90°C. The solutions turned quickly black and a monitoring of the reaction by TLC revealed that the reaction was accomplished. The solvent was then evaporated and the crude was purified by column chromatography on silica gel (Cyclohexane/EtOAc: 4/6) to obtain 582mg of 11 (90%) as a yellowish syrup. Rf=0.16 (Cyclohexane/EtOAc : 5/5). **1H-NMR** (300 MHz, CDCl₃): δ 9.83 (s, 1H, CHO), 7.82 (s, 1H, H triazol), 7.48-7.46 (m, 2H, H Ar), 6.84 (d, J = 8.6 Hz, 1H, H Ar), 5.34 (s, 2H, OCH₂), 5.19 (s, 2H, CH₂COOME),
4.12 (s, 4H, NCH$_2$), 3.79 (s, 3H, OMe), 3.60 (s, 6H, 2 OMe). $^{13}$C-NMR (75 MHz, CDCl$_3$): δ 190.25 (CHO), 170.94 (CO esters), 166.96 (CO ester), 148.78 (C Ar), 145.54 (C Ar), 134.82 (CH triazol), 132.47 (C Ar), 130.27 (C Ar), 127.69 (CH Ar), 117.43 (CH Ar), 112.24 (CH Ar), 59.57 (CH$_2$COOME), 53.65 (NCH$_3$), 53.08 (OMe), 52.05 (2 OMe), 49.62 (OCH$_2$). MS (Cl), calcd for C$_{18}$H$_{23}$N$_2$O$_6$ [M + H]$^+$ 435.1, found 435.2. HRMS (ES$^+$), calcd for C$_{18}$H$_{23}$N$_2$O$_6$ [M + H]$^+$ 435.1510, found 435.1526.

**Synthesis of X-Rhodamine : typical procedure**

**Numerotation of X-rhodamines :**

To a solution of aldehyde 5 (200 mg, 0.626 mmol) in propionic acid was added 8-hydroxyjulolidine (237 mg, 1.254 mmol, 2 eq) and PTSA (11 mg, 0.062 mmol, 0.1 eq). The solution was protected from light and stirred at room temperature overnight. The reaction mixture was added a solution of chloranil (152 mg, 0.626 mmol, 1 eq) in DCM (10 mL), the reaction turned dark and was allowed to stir overnight at room temperature. The dark purple solution was evaporated to dryness, dissolved in DCM. The crude was purified by column chromatography on silica gel (gradient of 100% DCM to 9/1 DCM/Methanol) to obtain 58 mg of 6 (∼13%) as a purple solid after lyophilsation (dioxane/water : 1/1). $^1$H-NMR (300 MHz, CDCl$_3$): δ 7.83 (d, $J = 8.1$ Hz, 2H, CH Ar PTSA counter ion), 7.05 (d, $J = 8.0$ Hz, 2H, CH Ar PTSA counter ion), 6.93 (t, $J = 7.6$ Hz, 5H, H$_a$, H$_b$, H$_c$, H$_d$), 4.70 (d, $J = 2.2$ Hz, 2H, CH$_2$O), 4.27 (s, 4H, NCH$_2$), 3.83 (s, 6H, 2 OMe), 3.55 (m, 8H, H$_a$, H$_b$), 3.02 (t, $J = 6.1$ Hz, 4H, H$_d$), 2.73 (t, $J = 6.0$ Hz, 4H, H$_e$), 2.57 (t, $J = 2.1$ Hz, 1H, C=CH), 2.27 (s, 3H, Me PTSA counter ion), 2.12-1.98 (m, 8H, H$_a$, H$_b$). $^{13}$C-NMR (75 MHz, CDCl$_3$): δ 171.73 (CO esters), 154.11 (C Ar), 152.22 (C Ar), 151.04 (C Ar), 148.20 (C Ar), 144.65 (C Ar), 141.08 (C Ar), 138.14 (C Ar), 128.15 (CH PTSA), 126.77 (C$_i$), 126.29 (CH PTSA), 125.35, 124.10 (CH Ar), 123.56, 118.09 (CH Ar), 116.10 (CH Ar), 112.75, 105.43, 78.00 (C=CH), 76.28 (C=CH), 56.78 (CH$_3$O), 53.87 (NCH$_2$), 52.15 (2 OMe), 50.95 (C$_1$ or C$_4$), 50.48 (C$_1$ or C$_4$), 27.73 (C$_i$), 21.29 (Me PTSA), 20.73 (C$_i$), 19.96 (C$_6$), 19.77 (C$_8$). MS (ES$^+$), calcd for C$_{46}$H$_{42}$N$_3$O$_6$ [M$^+$] 660.3, found 660.7. HRMS (ES$^+$), calcd for C$_{46}$H$_{42}$N$_3$O$_6$ [M$^+$] 660.3068, found 660.3079.

9 was obtained as a purple solid after lyophilsation with ∼20% yield. $^1$H-NMR (300 MHz, CDCl$_3$): δ 8.11 (s, 1H, H triazol), 7.71 (d, $J = 8.1$ Hz, 2H, 2CH PTSA), 6.95 (dd, $J = 4.8$, 3.0 Hz, 3H, CH PTSA, 1CH Ar), 6.86-6.73 (m, 4H, CH Ar), 5.22 (s, 2H, CH$_2$COOME), 5.11 (s, 2H, OCH$_2$), 4.16 (s, 4H, NCH$_2$), 3.68 (s, 3H, OMe), 3.65 (s, 6H, 2 OMe), 3.44 (m, 8H, H$_a$, H$_b$), 2.94 (t, $J = 6.1$ Hz, 4H, H$_d$), 2.69-2.64 (m, 4H, H$_e$), 2.18 (s, 3H, Me PTSA), 2.02 (t, $J = 5.1$ Hz, 4H, H$_j$), 1.93-1.91 (m, 4H, H$_j$). $^{13}$C-NMR (75 MHz, CDCl$_3$): δ 171.82 (CO esters), 166.99 (CO ester), 154.70 (C Ar), 152.26 (C Ar), 151.02 (C Ar), 149.12 (C Ar), 144.59 (C Ar), 142.88 (C Ar), 141.13 (C Ar), 138.21 (C Ar), 128.18 (CH PTSA), 127.03 (C$_i$),
126.23 (CH PTSA), 126.09 (CH triazol), 125.36 (C Ar), 123.63 (C Ar), 123.48 (CH Ar), 117.91 (CH Ar), 115.93 (CH Ar), 112.88 (C Ar), 105.24 (C Ar), 62.56 (OCH), 53.82 (NCH), 52.92 (OMe), 52.03 2 (OMe), 50.96 (C1 or C4), 50.79 (CH2COOME), 50.44 (C1 or C4), 27.64 (C3), 21.28 (Me PTSA), 20.74 (C2), 19.97(C6), 19.81 (C4). MS (ES+), calcd for C45H37N6O8 [M]+ 775.3450, found 776.0. HRMS (ES+), calcd for C45H37N6O8 [M]+ 775.3450, found 775.3473.

**Saponification : typical procedure**

To a solution of **12** (90 mg, ~0.10 mmol) in methanol (10 mL) were added 700 mg of KOH and water (3 mL), the mixture was stirred overnight. The product was washed with HCl (1M) and extracted with CHCl3 until the aqueous phase become slightly pink. The organic phase was then dried over MgSO4, filtered and evaporated. The crude was purified on a reverse phase column C-18 using acetonitrile (0,1% TFA) and water (0,1% TFA) as eluant (20% ACN to 60%), monitored at 254 nm. The solvents were evaporated and 64 mg of **13** (76%) were obtained as a purple solid after lyophilisation (dioxane/water, 1/1). HRMS (ES+), calcd for C45H37N6O8 [M]+ 733.2980, found 733.3002.

**7** was obtained as a purple solid after lyophilisation with ~74% yield. HRMS (ES+), calcd for C38H38N4O6 [M]+ 632.2755, found 632.2761.
10 was obtained as a purple solid after lyophilisation with ~81% yield. HRMS (ES⁺), calcd for C₄₀H₄₂N₆O₈ [M⁺] 733.2980, found 733.3002

Dextran Conjugates

Dextran 6,000 MW (Sigma-Aldrich, ref: 31388) and dextran 1,500 MW (Sigma-Aldrich, ref: 31394) were functionnalised with 14 using a method described by Nielsen et al. The ¹H-NMR showed that the functionnalised dextrans were alkylated once by glucose unit.

Conjugation of Dextrans. To a solution of dextran-PEG-N₃ (1,500 or 6,000) (40 mg, ~100 µmol glucose unit) and 7 (10 mg, 14 µmol) in DMF (2 mL) was added an heterogeneous solution of CuSO₄·5H₂O (10 mg, 40 µmol) and sodium ascorbate (10 mg, 50 µmol) in water (1 mL). The solution was allowed to stir in the dark at 50°C overnight. The solvents were evaporated, the crude was dissolved in 1 mL of EDTA solution (0.1 M) and passed through a G-25 column to give 40 mg of CaRu-Dextran 6,000 conjugate (~80% yield) and 38 mg CaRu-Dextran 1,500 conjugate (~76% yield).

Figure S1. Absorbance spectra and Emission spectra (λₑ= 535 nm) of 10 (5 mM, MOPS 30 mM, KCl 100 mM) in presence of 3 equivocation (15 mM) of various metals and EDTA (1 mM).

**Determination of the Dissociation constants Kd.**

The dissociation constants were obtained by fitting the Hill equation with the Plot of fluorescence enhancement vs. increasing concentration of Ca$^{2+}$, Hill equation is given below:

$$\theta = \frac{[L]^n}{K_d + [L]^n}$$

Where

$\theta$ is the fraction of the Ca$^{2+}$-binding sites on the receptor which are occupied by Ca$^{2+}$.

$[L]$ is the free probe concentration

$n$ is the Hill coefficient

**pKa determination:**

![Figure S2](image.png)

**Figure S2.** Plot of fluorescence enhancement of 10 (5 mM, MOPS 30 mM, KCl 100mM) vs. pH. Hill’s equation fitting provided the pKa.
**Figure S3.** Emission spectra (λex = 535 nm) and absorbance spectra of dextran conjugate 6000 (left) and 1500 (right) (MOPS 30 mM, KCl 100 mM) at increasing concentration of Ca\(^{2+}\). Bottom: Plot of fluorescence enhancement ([F-F0] / F0, with F0 = Fluorescence Intensity in presence of EDTA 1 mM) of dextran conjugate 6000 (left) and 1500 (right) (MOPS 30 mM, KCl 100 mM) vs. increasing concentration of Ca\(^{2+}\). The fit line, according to Hill’s equation, yielded the Kd.

**Figure S4.** Fluorescence emission spectra of 10 (left) and its dextran conjugates 1500 (middle) 6000 right (MOPS 30 mM, KCl 100 mM) in presence of Ca\(^{2+}\), Mg\(^{2+}\) and a mixture of both.
The fluorescence quantum yields $\phi$ of 7,10 and 13 (from left to right) were calculated from the slope of the integrated spectral emission ($545$ to $700$ nm) of the sensors and reference dye vs. absorbance using Rhodamine 101 ($\phi = 1.0$ in absolute ethanol) as a reference standard (excitation wavelength was $535$ nm). To avoid self-absorption, we worked with solutions of $OD$ 0.01–0.1. Equation 2 was used where $\phi$ is the quantum yield, $\eta$ is the refractive index ($\eta (H_2O) = 1.33$, $\eta (EtOH) = 1.36$) and $s$ is the value of the slope. The subscript $ref$ refers to the reference. Quantum yield determinations of the sensors were measured in a solution of $1$ mM EGTA (MOPS 30 mM, KCl 100 mM) and in a solution of $1$ mM Ca$^{2+}$ (MOPS 30 mM, KCl 100 mM).

\begin{equation}
\phi = \phi_{ref} \frac{s}{s_{ref}} \frac{\eta^2}{\eta_{ref}^2}
\end{equation}
NMR and Mass Spectra

$^1$H Spectrum of 2

$^{13}$C Spectrum of 2
$^1$H Spectrum of 6
$^1$H Spectrum of 12

$^{13}$C Spectrum of 12
$^1$H Spectrum of 14

$^1$H Spectra of Dextran 1,500-PEG-N$_3$ (D$_2$O)
H Spectra of Dextran 6,000-PEG-N₃ (D₂O)

^{1}H Spectra of Dextran 6,000-PEG-N₃ (D₂O)
HRMS Spectra of 2

Elemental Composition Report

Single Mass Analysis
Tolerance ± 10.0 PPM  /  DBE: min = -1.5, max = 80.0
Element prediction: Off
Number of isotope peaks used for: IPIT = 3

Vibrational Mass, Even Electron Ion
271 formulae evaluated with 12 results within 10 ppm (up to 1000 dozen results for each mass)
Elements Used:
C: 0.46  H: 0.100  N: 0.6  O: 0.6

292.1317  292.1319  0.1  0.3  12.5  40.0  2.7  156  114  95
292.1317  292.1319  0.1  -0.3  12.5  40.0  2.7  156  114  95
292.1317  292.1319  0.1  -0.3  12.5  40.0  2.7  156  114  95

HRMS Spectra of 4

Cmpd: C16 H17 N O6: +APCI Scan (0.259-0.267 min, 2 Scans) Frac=100.0V ENS_P020.d

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HRMS Spectra of 5
**Elemental Composition Report**

**Single Mass Analyse**

Tolerance = 10.0 PPM / DEE: min = -1.5, max = 80.0
Element prediction: Off
Number of isotope peaks used for FIT = 3

**Merokopane Mass, Even Electron ions**

415 formula units evaluated with 4 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 0.40  H: 0.100  N: 0.0  O: 0.0
LCT Premier XE MS63

**HRMS Spectra of 8**

**Elemental Composition Report**

**Single Mass Analyse**

Tolerance = 10.0 PPM / DEE: min = -1.5, max = 80.0
Element prediction: Off
Number of isotope peaks used for FIT = 3

**Merokopane Mass, Even Electron ions**

415 formula units evaluated with 5 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 0.40  H: 0.100  N: 0.0  O: 0.0
LCT Premier XE MS63

**HRMS Spectra of 11**

**Elemental Composition Report**

**Single Mass Analyse**

Tolerance = 10.0 PPM / DEE: min = -1.5, max = 80.0
Element prediction: Off
Number of isotope peaks used for FIT = 3

**Merokopane Mass, Even Electron ions**

505 formula units evaluated with 5 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 0.40  H: 0.100  N: 0.0  O: 0.0
LCT Premier XE MS63

**HRMS Spectra of 6**
HRMS Spectra of 9

HRMS Spectra of 12

HRMS Spectra of 7
HRMS Spectra of 13