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# Electronic Supplementary Information

for

# A far-red fluorescent probe based on a phospha-fluorescein scaffold for cytosolic calcium imaging

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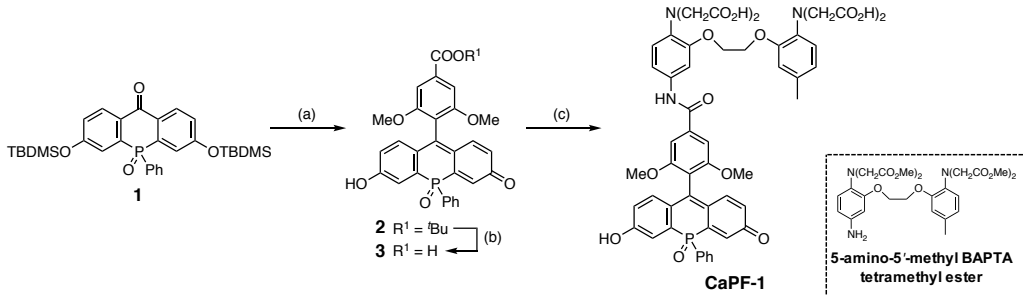
# Contents

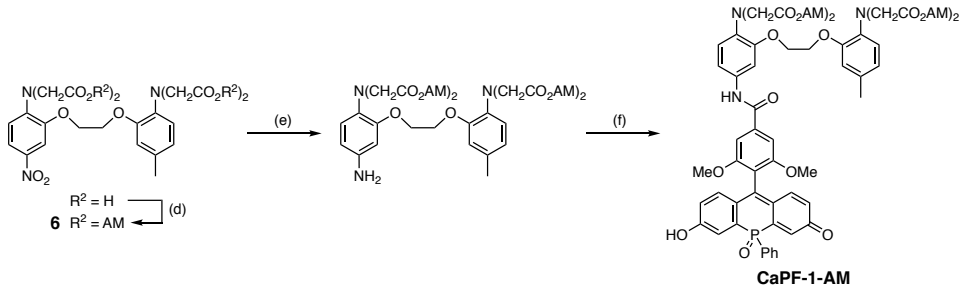
## 1. Experimental Details

**General.** $^{1}H$, $^{13}C\{^{1}H\}$, and $^{31}P\{^{1}H\}$ NMR spectra were recorded with a JEOL ECZ 400 spectrometer equipped with a SuperCOOL probe in $CDCl_3$ or methanol-$d_4$ (400 MHz for $^{1}H$, and 162 MHz for $^{31}P$). $^{13}C\{^{1}H\}$ NMR spectra (150 MHz) of the compounds were recorded with a JEOL ECA 600 II spectrometer equipped with an UltraCOOL probe. The chemical shifts in $^{1}H$ NMR spectra are reported in $\delta$ ppm using the signals of $CHCl_3$ (7.26 ppm) or methanol (3.31 ppm) as an internal standard and those in $^{13}C$ NMR spectra are reported using the solvent signals of $CDCl_3$ (77.16 ppm), methanol-$d_4$ (49.00 ppm), DMSO-$d_6$ (39.52 ppm) as an internal standard. The chemical shifts in $^{31}P$ NMR spectra are reported using $H_3PO_4$ (0.00 ppm) as an external standard. Mass spectra were measured with an Exactive Mass Spectrometer (Thermo scientific) with the ionization methods of electrospray ionization (ESI). Reversed phase column chromatography was performed using LC-Forte/R (YMC) equipped with a reversed phase silica gel column (YMC-DispoPack AT ODS 12g, YMC). Preparative reversed phase HPLC was performed on an YMC Triart C18 (10 × 250 mm) column (YMC) using Delta 600 system (Waters). Anhydrous dichloromethane, THF, and DMF were purchased from Kanto Chemicals and further purified by Glass Contour Solvent Systems. 5-Methyl-5′-nitro BAPTA,[1] 5-amino-5′-methyl BAPTA tetramethyl ester,[2] and bis(*t*-butyldimethylsiloxy)-phospha-xanthone **1**,[3] and POF-$Me_2$[4] were prepared according to the literature methods. All other chemicals were purchased from commercial suppliers and used without further purification.

A. Phospha-fluorescein based calcium probe **CaPF-1**




B. Membrane permeable **CaPF-1-AM**




**Scheme. S1** Synthesis of **CaPF-1** and its acetoxymethyl ester **CaPF-1-AM**. *Reagents and conditions*: (a) i) 4-*tert*-butoxycarbonyl-2,6-dimethoxyphenyllithium, THF, –78 °C then rt, ii) 10% TFA, $CH_2Cl_2$, rt, 50 min, 63% for 2 steps; (b) 50% TFA, $CH_2Cl_2$, rt, 1 h, 74%; (c) i) 5-amino-5′-methyl BAPTA tetramethyl ester, HATU, HOBt·$H_2O$, DMF, 40 h, ii) MeOH, NaOH aq. 4.5 h, 17% for 2 steps; (d) bromomethyl acetate, Et(*i*Pr)$_2$N, $CH_2Cl_2$, 3 d, 49%; (e) $H_2$, Pd/C, EtOAc, rt, 25 h, 54%; (f) **3**, 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluoro-phosphate (HATU), 1,2,3-benzotriazol-1-ol monohydrate (HOBt·$H_2O$), DMF, 15 h, 56% (based on **3**).

***tert*-Butyl 4-bromo-3,5-dimethoxybenzoate.**[5] 4-Bromo-3,5-dimethoxybenzoic acid (5.22 g, 20.0 mmol), DCC (5.36 g, 26.0 mmol) and DMAP (244 mg, 2.00 mmol) were dissolved in 60 mL of dichloromethane under a nitrogen atmosphere. The solution of *tert*-butanol (3.85 g, 52.0 mmol) in 20 mL of dichloromethane was added and the resulting mixture was stirred for 3 days at room temperature. The white precipitate was removed by filtration and the filtrate was concentrated under reduced pressure. The mixture was purified by silica gel column chromatography (hexane/$CH_2Cl_2$ 3/2 to 1/1) to give 3.34 g (10.5 mmol, 53%) of **4** as white solid. $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 7.20 (s, 2H), 3.95 (s, 6H), 1.61 (s, 9H). $^{13}$C{$^1$H} NMR (125 MHz, $CDCl_3$) $\delta$ 165.3, 157.0, 132.2, 106.3, 105.6, 81.9, 56.7, 28.3.

**POF *tert*-butyl ester 2**. To a solution of *tert*-butyl 4-bromo-3,5-dimethoxybenzoate (567 mg, 1.79 mmol) in anhydrous THF (8.0 mL) was added *sec*-BuLi (1.05 M in hexane and cyclohexane, 1.6 mL, 1.68 mmol) at –78 °C over 13 min and the mixture was stirred for 3 h at the same temperature. A solution of bis(*t*-butyldimethylsiloxy)-phospha-xanthone **1** (203 mg, 0.360 mmol) in anhydrous THF (2.0 mL) was added dropwise at –78 °C over 13 min. The reaction mixture was allowed to warm to room temperature and further stirred for 17 h. After removal of the solvent under reduced pressure, 5.0 mL of a 10/1 $CH_2Cl_2$/TFA mixed solution was added to the mixture followed by stirring for 50 min at room temperature. An aqueous solution of NaOH was added to the solution and the mixture was extracted with ethyl acetate three times. The combined organic layer was washed with 1 M HCl aqueous solution and brine, dried over $Na_2SO_4$, filtered, and concentrated under reduced pressure. The obtained red solid was subjected to reversed-phase column chromatography (C18) eluted with a linear gradient of 20–40% $CH_3CN$ in an aqueous solution of 5 mM $(NH_4)_2CO_3$. The fraction was acidified with 2 M HCl aqueous solution and extracted with ethyl acetate three times. The combined organic layers were washed with brine, dried over $Na_2SO_4$, filtered, and concentrated under reduced pressure to afford 127 mg (0.228 mmol, 63 %) of **2** as a red solid. $^1$H NMR (400 MHz, $CD_3OD$): $\delta$ 7.79–7.71 (m, 2H), 7.59 (dt, $J$ = 1.9, 8.2 Hz, 1H), 7.56–7.48 (m, 2H), 7.43 (dd, $J$ = 1.2, 9.2 Hz, 2H), 7.23 (d, $J$ = 2.4 Hz, 1H), 7.19 (d, $J$ = 2.4 Hz, 1H), 7.06 (dd, $J$ = 6.2, 9.2 Hz, 2H), 6.59 (dd, $J$ = 1.8, 11.6 Hz, 2H), 3.82 (s, 3H), 3.81 (s, 3H), 1.66 (s, 9H). $^{13}$C{$^1$H} NMR (150 MHz, $CD_3OD$): $\delta$ 166.4 (s, C), 159.2 (s, C), 158.8 (s, C), 150.8 (d, $J_{CP}$ = 8.6 Hz, C), 139.0 (br, C), 137.5, (d, $J_{CP}$ = 89.0 Hz, C), 136.5 (s, C), 133.98 (s, CH), 133.97 (d, $J_{CP}$ = 109.1 Hz, C), 131.1 (d, $J_{CP}$ = 11.4 Hz, CH), 130.3 (d, $J_{CP}$ = 12.9 Hz, CH), 127.7 (d, $J_{CP}$ = 5.7 Hz, C), 125.2 (br, CH), 118.9 (s, C), 106.0 (s, CH), 105.9 (s, CH), 83.1 (s, C), 56.8 (s, $CH_3$), 56.7 (s, $CH_3$), 28.4 (s,

$CH_3$). One aromatic CH peak is overlapped. $^{31}P\{^1H\}$ NMR (162 MHz, $CD_3OD$): $\delta$ 10.2. HRMS (ESI): *m*/*z* calcd. for $C_{32}H_{29}O_7PNa$: 579.1549 ($[M+Na]^+$); found. 579.1541.

**POF carboxylic acid 3**. A solution of **2** (18.5 mg, 33.2 $\mu$mol) in a 1/1 (v/v) mixture of $CH_2Cl_2$/TFA (2.0 mL) was stirred at room temperature for 1 h. All the volatiles were removed under reduced pressure. The obtained red solid was subjected to reversed phase HPLC (YMC-Triart C18, 10 × 250 mm) using a linear gradient of 20–80% $CH_3CN$ in water containing 0.1% TFA. The fraction was lyophilized to give 12.3 mg (24.6 $\mu$mol, 74%) of **3** as a red powder. $^1H$ NMR (400 MHz, $CD_3OD$): $\delta$ 7.80–7.71 (m, 2H), 7.65–7.57 (m, 1H), 7.57–7.49 (m, 4H), 7.21 (br d, *J* = 14.8 Hz, 2H), 7.08 (dd, *J* = 6.4, 9.6 Hz, 2H), 6.61 (br s, 2H), 3.83 (s, 3H), 3.82 (s, 3H). $^{13}C\{^1H\}$ NMR (150 MHz, DMSO-$d_6$): $\delta$ 166.8 (s, C), 157.9 (d, *J* = 35.9 Hz, C), 157.4 (s, C), 157.0 (s, C), 147.3 (d, *J* = 5.9 Hz, C), 134.0 (d, *J* = 106.4 Hz, C), 133.9 (s, C), 132.4 (s, CH), 129.4 (d, *J* = 10.1 Hz, CH), 129.2 (d, *J* = 12.9 Hz, CH), 120.3 (br, CH), 118.3 (br, CH), 117.3 (s, C), 116.3 (br, CH), 105.2 (d, *J* = 8.7 Hz, CH), 56.5 (s, $CH_3$), 56.2 (s, $CH_3$). One aromatic C peak and one aromatic CH peak are overlapped). $^{31}P\{^1H\}$ NMR (162 MHz, $CDCl_3$): $\delta$ 10.2. HRMS (ESI): *m*/*z* calcd. for $C_{28}H_{20}O_7P$: 499.0947 ($[M-H]^-$); found. 499.0949.

**CaPF-1**. A solution of **3** (23.5 mg, 47.0 $\mu$mol), 5-amino-5′-methyl BAPTA tetramethyl ester (134 mg, 239 $\mu$mol), HATU (317 mg, 833 $\mu$mol), and HOBt·$H_2O$ (167 mg, 1.24 mmol) in anhydrous DMF (9.0 mL) was stirred for 40 h at room temperature. After removal of the solvent, the mixture was subjected to reversed phase column chromatography (YMC-DispoPack AT ODS 12g) using a linear gradient of 20–80% $CH_3CN$ in water containing 0.1% TFA. The fractions containing the target compound were collected and concentrated under reduced pressure followed by addition of water and extraction with ethyl acetate three times. The combined organic layer was washed with brine, dried over $Na_2SO_4$, filtered, and concentrated under reduced pressure. The obtained red solid was dissolved in a mixture of THF (1.5 mL) and 1 M $NaOH_{aq}$ (6.0 mL) and the resulting solution was stirred for 4.5 h at room temperature. The reaction mixture was acidified with 1 M HCl aqueous solution and extracted with ethyl acetate four times. The combined organic layers were concentrated under reduced pressure. The crude mixture was subjected to reversed phase column (YMC-DispoPack AT ODS 12 g) using a linear gradient of 20–100% $CH_3CN$ in water containing 0.1% TFA. The lyophilized compound was further purified with reversed phase HPLC (YMC-Triart C18, 10 × 250 mm) eluted with a linear gradient system of 20–80% $CH_3CN$ in water containing 0.1% TFA. The fraction was

lyophilized to afford 8.1 mg (8.20 $\mu$mol, 17%) of **CaPF-1** as a red solid. $^{1}$H NMR (400 MHz, $CD_3OD$): $\delta$ 7.76 (dd, $J$ = 7.2, 13.2 Hz, 2H), 7.61 (t, $J$ = 7.2 Hz, 1H), 7.58–7.51 (m, 3H), 7.46 (d, $J$ = 8.0 Hz, 2H), 7.29 (d, $J$ = 9.2 Hz, 1H), 7.24 (d, $J$ = 2.0 Hz, 1H), 7.20 (d, $J$ = 2.0 Hz, 1H), 7.11 (dd, $J$ = 6.2, 9.4 Hz, 2H), 7.02 (d, $J$ = 8.4 Hz, 1H), 6.90–6.86 (m, 2H), 6.71 (d, $J$ = 8.0 Hz, 1H), 6.61 (d, $J$ = 9.2 Hz, 2H), 4.50–4.35 (m, 4H), 4.07 (s, 4H), 4.01 (s, 4H), 3.87 (s, 3H), 3.86 (s, 3H), 2.30 (s, 3H), $^{13}$C{$^{1}$H} NMR (150 MHz, $CD_3OD$): $\delta$ 175.8 (s, C), 175.6 (s, C), 167.7 (s, C), 159.3 (s, C), 159.0 (s, C), 152.1 (s, C), 152.0 (s, C), 150.8 (d, $J$ = 7.2 Hz, C), 139.6 (s, C), 139.0 (br, CH), 137.7 (s, C), 137.6 (br, CH), 137.4 (s, C), 134.8 (d, $J$ = 54.6 Hz, C), 134.0 (s, CH), 133.9 (d, $J$ = 109.2 Hz, C), 131.1 (d, $J$ = 10.1 Hz, CH), 130.3 (d, $J$ = 13.1 Hz, CH), 128.0 (s, C), 127.8 (s, CH), 125.1 (br, CH), 122.8 (s, CH), 120.5 (s, CH), 120.2 (s, CH), 117.8 (s, C), 115.4 (d, $J$ = 5.7 Hz, CH), 108.7 (s, C), 104.7 (s, CH), 104.6 (s, CH), 101.3 (s, C), 68.5 (s, $CH_2$), 68.1 (s, $CH_2$), 57.0 (s, $CH_3$), 56.8 (s, $CH_3$), 56.1 (s, $CH_2$), 55.8 (s, $CH_2$), 21.1 (s, $CH_3$). One aromatic CH carbon are overlapped. $^{31}$P{$^{1}$H} NMR (162 MHz, $CD_3OD$): $\delta$ 10.2. HRMS (ESI): $m/z$ calcd. for $C_{51}H_{45}N_3O_{16}P$: 986.2537 ([$M$–H]$^-$); found. 986.2501.

**Compound 6.** A solution of 5-methyl-5′-nitro BAPTA (136 mg, 0.254 mmol), acetoxymethyl bromide (0.14 mL, 1.52 mmol), and diisopropylethylamine (0.27 mL, 1.52 mmol) in dry dichloromethane (3.0 mL) was stirred at room temperature for 3 d under a nitrogen atmosphere. After addition of 0.1 M HCl aqueous solution, the organic layer was separated, and the aqueous layer was extracted with dichloromethane two times. The combined organic layer was washed with brine, dried over anhydrous $Na_2SO_4$, filtered, and concentrated under reduced pressure. The obtained mixture was subjected to reversed phase column chromatography (YMC-DispoPack AT ODS 12g) using a linear gradient of 20–100% $CH_3CN$ in water containing 0.1% TFA. The fraction was lyophilized to afford 102 mg (0.123 mmol, 49%) of **6** as yellow viscous oil. $^{1}$H NMR (400 MHz, $CDCl_3$): $\delta$ 7.85 (dd, 1H, $J$ = 8.6, 2.6 Hz), 7.78 (d, 1H, $J$ = 2.6 Hz), 6.84 (d, 1H, $J$ = 8.0 Hz), 6.77–6.69 (m, 3H), 5.65 (s, 4H), 5.62 (s, 4H), 4.41–4.26 (m, 8H), 4.14 (s, 4H), 2.28 (s, 3H), 2.08 (s, 6H), 2.07 (s, 6H). $^{13}$C NMR (150 MHz, $CDCl_3$): $\delta$ 170.2, 169.6, 169.5, 169.4, 150.6, 149.0, 144.8, 141.6, 136.5, 133.3, 122.6, 120.6, 118.3, 116.7, 115.2, 108.4, 79.7, 79.3, 68.0, 67.0, 53.7, 53.5, 21.1, 20.8. One methyl peak is overlapped. HRMS (ESI): $m/z$ calcd. for $C_{35}H_{41}N_3O_{20}Na$: 846.2181, ([$M$+Na]$^+$); found 846.2167.

**Compound 5.** A suspension of **6** (50.4 mg, 61.2 $\mu$mol) and 10% Pd/C(14.3 mg, 14.1 $\mu$mol) in ethyl acetate (10 mL) was stirred at room temperature for 25 h under a hydrogen atmosphere.

The reaction mixture was filtered through the plug of glass filter, and the filtrate was concentrated under reduced pressure. The obtained mixture was subjected to reversed phase column chromatography (YMC-DispoPack AT ODS 12g) using a linear gradient of 20–80% $CH_3CN$ in water containing 0.1% TFA. The fraction was lyophilized to afford 30.1 mg (33.2 $\mu$mol, 54%) of **5** as a TFA salt (colorless solid). $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 7.05 (br s, 1H), 6.92 (br d, $J$ = 7.2Hz, 1H), 6.84–7.78 (m, 2H), 7.73–6.68 (m, 2H), 5.94 (br s, 2H), 5.60 (s, 4H), 5.59 (s, 4H), 4.34–4.18 (m, 4H), 4.13 (s, 4H), 4.10 (s, 4H), 2.27 (s, 3H), 2.06 (s, 6H), 2.03 (s, 6H). $^{13}$C{$^1$H} NMR (150 MHz, $CDCl_3$): $\delta$ 170.6, 170.1, 170.0, 169.7, 151.1, 150.5, 138.8, 135.8, 133.7, 125.9, 122.2, 120.2, 120.1, 115.9, 114.2, 108.9, 79.53, 79.47, 67.9, 66.9, 53.7, 53.5, 21.1, 20.73, 20.69. HRMS (ESI): $m/z$ calcd. for $C_{35}H_{43}N_3O_{18}Na$: 816.2439 ([$M$+Na]$^+$); found 816.2427.

**CaPF-1-AM**. A solution of **3** (26.1 mg, 52.2 $\mu$mol), **5** (125 mg, 138 $\mu$mol), HATU (101 mg, 265 $\mu$mol), and HOBt·$H_2O$ (35.8 mg, 265 $\mu$mol) in anhydrous DMF (1.5 mL) was stirred for 15 h at room temperature. After addition of 0.2 M HCl aqueous solution, the aqueous layer was separated and extracted with ethyl acetate three times. The combined organic layer was washed with 0.2 M HCl aqueous solution, brine, dried over $Na_2SO_4$, filtered, and concentrated under reduced pressure. The crude mixture was subjected to reversed phase column (YMC-DispoPack AT ODS 12g) where the eluent was gradually changed from 40% $CH_3CN$ to 100% $CH_3CN$ with 0.1% TFA. The fraction containing CaPF-1-AM was concentrated under reduced pressure followed by addition of water and extracted with ethyl acetate three times. The combined organic layer was washed with brine, dried over $Na_2SO_4$, filtered, and concentrated under reduced pressure. The obtained red solid was further purified with reversed phase HPLC, where the eluent was gradually changed from 40% $CH_3CN$ to 100% $CH_3CN$ with 0.1% TFA. Lyophilization of the obtained product gave 37.5 mg (29.4 $\mu$mol, 56%) of CaPF-1-AM as a red powder. $^1$H NMR (400 MHz, methanol-$d_6$) $\delta$ 7.76 (dd, $J$ = 6.6 Hz, 12.8 Hz, 2H), 7.61 (t, $J$ = 6.6 Hz, 1H), 7.57-7.51 (m, 3H), 7.45 (d, $J$ = 8.0 Hz, 2H), 7.30–7.17 (m, 3H), 7.11 (dd, $J$ = 6.2, 9.4 Hz, 2H), 6.93 (d, $J$ = 8.0 Hz, 1H), 6.84 (s, 1H), 6.78 (d, $J$ = 8.0 Hz, 1H), 6.69 (d, $J$ = 8.0 Hz, 1H), 6.61 (br, 2H), 5.63 (s, 4H), 5.62 (s, 4H), 4.41–4.31 (m, 4H), 4.21 (s, 4H), 4.16 (s, 4H), 3.87 (s, 3H), 3.86 (s, 3H), 2.28 (s, 3H), 2.05 (s, 6H), 2.02 (s, 6H). $^{31}$P{$^1$H} NMR (162 MHz, $CDCl_3$): $\delta$ 10.1. HRMS (ESI): $m/z$ calcd. for $C_{63}H_{61}N_3O_{24}P$: 1274.3383 ([$M$–H]$^-$); found. 1274.3368.

## 2. Photophysical Properties

**Photophysical measurements.** HEPES buffer solutions (50 mM, pH 7.4, 50 mM KCl) containing 1 mM $Ca(NO_3)_2$ or 1 mM EGTA were prepared. UV-vis absorption spectra of 10 $\mu$M CaPF-1 in HEPES buffers (prepared from a stock solution of 10 mM CaPF-1 in DMSO) were recorded using a Shimadzu UV-3150 spectrometer with a resolution of 1.0 nm (Fig. S1). Emission spectra of 1 $\mu$M CaPF-1 in HEPES buffers (prepared from a stock solution of 1 mM CaPF-1 in DMSO) were recorded using HORIBA FluoroMax-4 spectrometer with a resolution of 1.0 nm (Fig. S1). Absolute fluorescence quantum yields were determined with a Hamamatsu photonics PMA-11 calibrated integrating sphere system.

**Determination of calcium dissociation constant ($K_d$).** A series of HEPES-buffered solutions (50 mM, pH 7.4, 50 mM KCl, 0.1% DMSO, 1 $\mu$M CaPF-1) containing various amounts of $Ca(NO_3)_2 \cdot 4H_2O$ (ranging between 0 mM to 10 mM) and 10 mM EGTA were prepared. The concentration of free $Ca^{2+}$ ($[Ca^{2+}]_{free}$) was calculated using literature method.[6] The fluorescence intensity at 663 nm ($I_{663}$) was plotted against $[Ca^{2+}]_{free}$ and the experimental data were analyzed by non-linear least square curve fitting using the following equation (eq 1):

$$I_{663} = \frac{I_0 K_d + I_\infty [Ca^{2+}]_{free}}{K_d + [Ca^{2+}]_{free}} \qquad \text{(eq 1)}$$

where $I_0$ and $I_\infty$ represent the initial and final fluorescence intensities at 663 nm, respectively (Fig. S2).

**Determination of acid dissociation constant (p$K_a$).** A series of pH-buffered solutions was prepared with 0.1 M citric acid/0.1 M sodium citrate for pH 4–5.5 and 0.1 M $Na_2HPO_4$/0.1 M $NaH_2PO_4$ for pH 5.8–8.2. 2 $\mu$L of a stock solution of CaPF-1 in DMSO (10 mM) was added to each pH solution (2 mL) and the absorption spectra were measured (Fig. 2b). The absorbance at 635 nm ($A_{635}$) was plotted against the pH values. The data was analyzed by curve fitting using the following equation (eq 2):

$$A_{635} = \frac{10^{-pH} A_0 + 10^{-pKa} A_\infty}{10^{-pH} + 10^{-pKa}} \qquad \text{(eq 2)}$$

where $A_0$ and $A_\infty$ represent the initial and final absorbance values at 635 nm, respectively (Fig. S3).

**pH dependent fluorescence properties.** A series of pH-buffered solution (50 mM KCl) was prepared with 50 mM MES for pH 5.5–6.5 and 50 mM HEPES for pH 7.0–9.0 with (1 mM $Ca(NO_3)_2$) or without $Ca^{2+}$ (10 mM EGTA). 2 $\mu$L of a stock solution of CaPF-1 in DMSO (1 mM) was added to each pH solution (2 mL) and the emission spectra were measured. The relative fluorescence intensities ($F/F_0$) were plotted against the pH value (Fig. S4). The fluorescence quantum yields of CaPF-1 in pH 4.0 and 7.4 solutions containing 1 mM $Ca(NO_3)_2$ or 0.5 mM EGTA ($Ca^{2+}$-free) were measured (Table 1 and Table S1).

**Metal ion selectivity.** HEPES buffer solutions (50 mM, pH 7.4) of 1 $\mu$M CaPF-1 containing 1 mM $Ca(NO_3)_2$ or 0.5 mM EGTA ($Ca^{2+}$-free) were prepared. The pH values were adjusted by adding $Me_4NOH{\cdot}5H_2O$. To each solution, NaCl (100 mM), KCl (10 mM), $MgCl_2$ (10 mM and 1 mM), or $ZnCl_2$ (0.6 mM) was added and the emission spectra were recorded.

**Chemical stability in 10 mM GSH solution.** HEPES buffer solutions (50 mM, pH 7.4) of 1 $\mu$M CaPF-1 containing 10 mM GSH (reduced form) were prepared. The time course of absorption spectra in the solution were recorded (Fig. S6a). The relative absorbance at 635 nm ($A/A_0$) was plotted against each time point (Fig. S6b).

### 3. Electrochemical Properties

**Cyclic voltammetry.** Cyclic voltammetry (CV) was performed on an ALS/chi-617A electrochemical analyzer. The CV cell consisted of a glassy carbon electrode, a Pt wire counter electrode, and an Ag/AgCl (in 3 M NaCl) reference electrode (0.21 V vs. SHE). A solution of 5 mM POF-$Me_2$ in 0.1 M phosphate buffer (pH 10.0) was prepared and nitrogen gas was bubbled for 3 min before measurement (Fig. S7).

### 4. Cell Culture Experiments

HeLa cells (RIKEN Cell Bank, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) containing 10% fetal bovine serum (FBS, Gibco) and 1% Antibiotic-Antimycotic (AA, Sigma) at 37 °C in a 5% $CO_2$/95% air incubator. Cells ($5 \times 10^4$) were seeded in glass-bottom dishes three days before imaging. Immediately before use, a

solution of 10 mM CaPF-1-AM in DMSO was mixed with the 20 wt% Pluronic F-127 solution in DMSO at a ratio of 1:4. The pre-mixed solution was then diluted with HBSS to prepare a final CaPF-1-AM concentration of 2 $\mu$M. The solution was then vortexed for 1 min and centrifuged (20 min, 15000 rpm). The supernatant was used for cell staining.

**Co-staining experiments.** The incubation medium was removed from the cells, and the cells were further incubated with 2 $\mu$M CaPF-1-AM and 0.2 $\mu$M Calcein-AM in HBSS containing 0.2% DMSO and 0.04% Pluronic F-127 for 30 min in a $CO_2$ incubator. After cells were rinsed with HBSS three times and the dish was filled with 2 mL of HBSS, fluorescence images were obtained with a FV10i-DOC confocal laser-scanning microscope (OLYMPUS) (Fig. S8), where LD lasers of 635 nm and 473 nm were used for the excitation of CaPF-1 and Calcein, respectively. The cells were also co-stained with ER Tracker Red (Invitrogen) in the similar manner for Calcein except the excitation wavelength of 559 nm (Fig. S9).

**Multi-color imaging experiments.** After removal of the incubation medium from the culture dish, the cells were washed with HBSS three times. The cells were simultaneously stained with 2 $\mu$M CaPF-1-AM, 0.2 $\mu$M Hoechst (Dojindo), and 0.2 $\mu$M MitoTracker Green FM (Invitrogen) in HBSS containing 0.2% DMSO and 0.04% Pluronic F-127 for 30 min at 37 °C. After the incubation medium was removed, the cells were rinsed with HBSS three times and the dish was filled with 2 mL of HBSS. For multi-color imaging, a confocal microscope (TCS SP8 STED 3X; Leica) equipped with a 20x objective lens and a HyD detector was used. The confocal fluorescence images were obtained under the excitation at 405 nm (diode laser), 473 nm (white light laser), and 633 nm (white light laser) for Hoechst ($\lambda_{em}$ = 420–460 nm), MitoTracker Green FM ($\lambda_{em}$ = 490–540 nm), and CaPF-1 ($\lambda_{em}$ = 638–795 nm), respectively. Each image was recorded with a line average of 4 and a frame average of 4 (Fig. 3).

**Fluorescence imaging of histamine-induced $Ca^{2+}$ oscillation in HeLa cells.** The incubation medium was removed from the dish and the cells were incubated with 2 $\mu$M CaPF-1-AM in HBSS containing 0.2% DMSO and 0.04% Pluronic F-127 for 1 h. The cells were rinsed with HBSS three times, filled with 2 mL of HBSS, and further incubated for 30 min in a $CO_2$ incubator. For histamine stimulation, 1 mL of histamine dihydrochloride (Wako) in HBSS (3 $\mu$M) was added to the medium (2 mL) to give the final concentration of 1 $\mu$M. For fluorescent time-lapse imaging, a confocal microscope (TCS SP8 STED 3X; Leica) equipped with a 20x

objective lens was used. The CaPF-1 was excited with 633 nm (white light laser) and the fluorescence was collected between 638-795 nm with a HyD detector (gain = 50) using a resonant scanning mode at 12 kHz. Each image was obtained from 6-line average and 3 times frame accumulation and the frame rate is 1.04 s. The images were processed with Fiji software.

**Cytotoxicity evaluation.** HeLa cells were seeded into a flat-bottomed 96-well plate ($1 \times 10^4$ cells/well) and incubated in DMEM containing 10% FBS at 37 °C in a 5% $CO_2$/95% air incubator for 24 h. The medium was then replaced with a culture medium containing various concentrations of CaPF-1-AM (0, 1, 2, 5, and 10 $\mu$M) in HBSS (0.4% DMSO and 0.04% Pluronic F-127). After the cells were incubated for 3 h at 37 °C, the medium was removed and the cells were washed with PBS three times. MTT reagent (final concentration, 0.5 mg/mL) in PBS was added to each well, and the plates were incubated for another 4 h in a $CO_2$ incubator. Excess MTT tetrazolium solution was then removed and the cells were once washed with PBS. After the formazan crystals were solubilized in DMSO (100 $\mu$L/well) for 30 min at room temperature, the absorbance of each well was measured by SpectraMax i3 (Molecular Devices) with an excitation at 535 nm (Fig. S10).

## 5. References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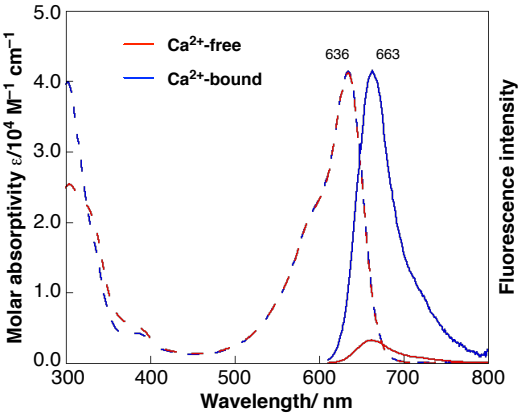


**Fig. S1** Absorption (dashed line; 0.5% DMSO) and emission (solid line; 0.1% DMSO) spectra of CaPF-1 in 50 mM HEPES buffer (pH 7.4). Red and blue lines represent the spectra of $Ca^{2+}$-free and $Ca^{2+}$-bound forms observed with the presence of 0.5 mM EGTA and 1 mM $Ca^{2+}$, respectively. The emission spectra were measured with the excitation wavelength at 580 nm.

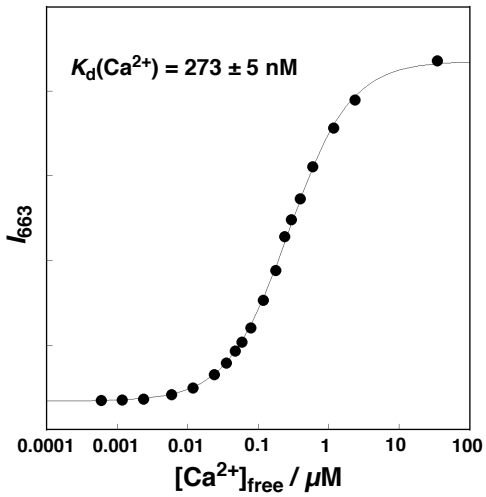


**Fig. S2** Plots of the fluorescence intensity at 663 nm ($I_{663}$) as a function of $[Ca^{2+}]_{free}$ with a best-fit curve for the dissociation constant of $K_d$ = 273 ± 5 nM.

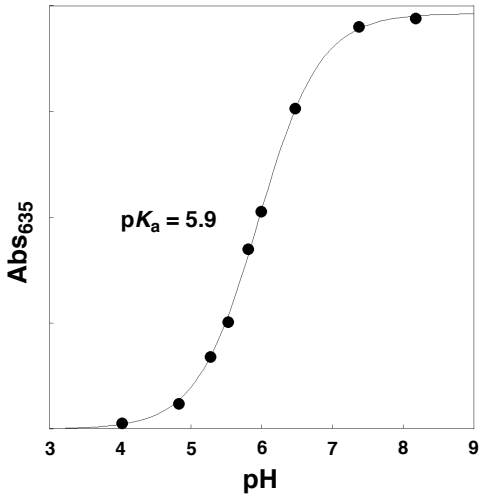


**Fig. S3** Plots of the absorbance at 635 nm ($Abs_{635}$) as a function of pH values with a best-fit curve for the acid dissociation constant of p$K_a$ = 5.9.

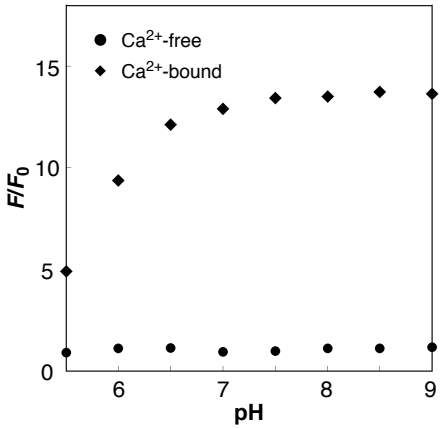


**Fig. S4** Plots of the fluorescence intensity ratio ($F/F_0$) at 663 nm as a function of pH values for 1 $\mu$M CaPF-1. Closed circle and diamond represent the $Ca^{2+}$-free and $Ca^{2+}$-bound forms, respectively.

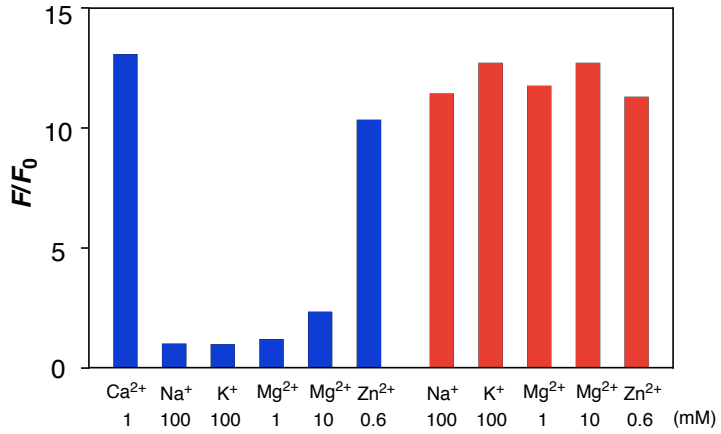


**Fig. S5** Fluorescence response of CaPF-1 (1 $\mu$M) to various metal ions (100 mM $Na^+$, 100 mM $K^+$, 1 mM $Mg^{2+}$, 10 mM $Mg^{2+}$, or 0.6 mM $Zn^{2+}$) in 0.1 M HEPES buffer (pH 7.4) containing 0.5 mM EGTA. Blue and red bars represent the fluorescence intensity ratio ($F/F_0$) of CaPF-1 in the presence and absence of 1 mM $Ca(NO_3)_2$, respectively.

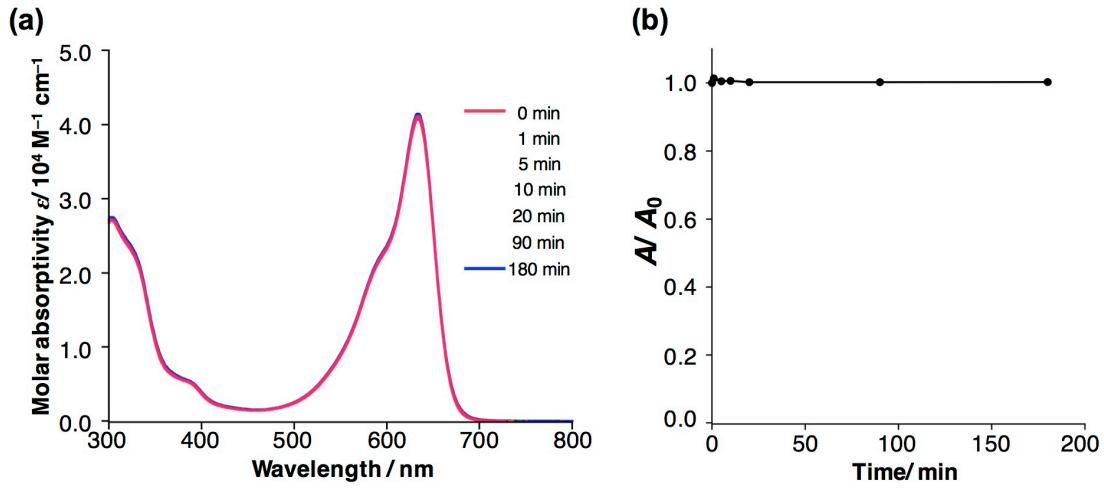


**Fig. S6** (a) Absorption spectral change of CaPF-1 (1 $\mu$M) in 0.1 M HEPES buffer (pH 7.4) containing 10 mM GSH (reduced form). (b) Plot of relative absorbance ($A/A_0$) at 635 nm versus each time point.

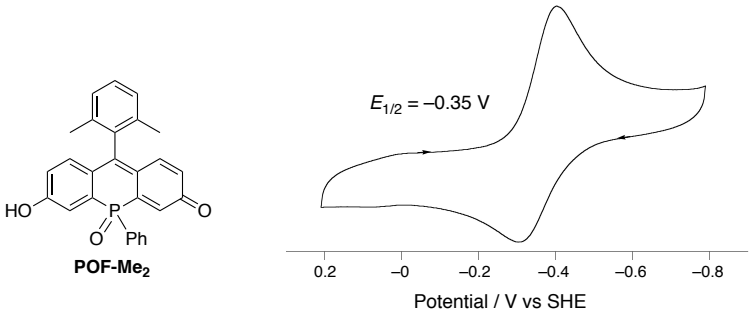


**Fig. S7** Cyclic voltammogram of POF-$Me_2$ at a scan rate of 0.1 V $s^{-1}$ in pH 10 phosphate buffer. The potential vs. Ag/AgCl (in 3 M NaCl) was converted to vs. SHE by adding 0.21 V.

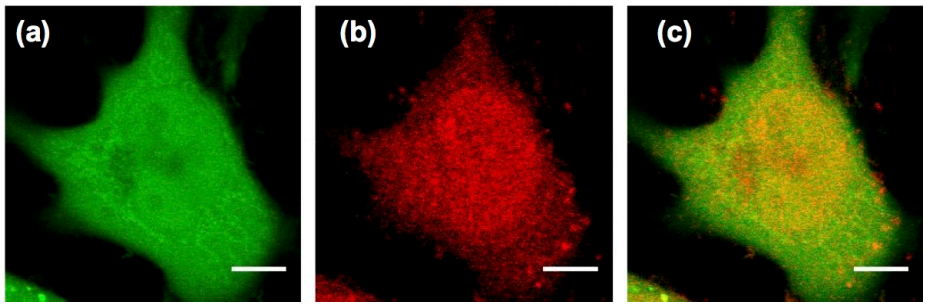


**Fig. S8** Co-staining experiment of HeLa cells with Calcein and CaPF-1. The cells were loaded with Calcein-AM (0.2 $\mu$M) and CaPF-1-AM (2 $\mu$M) at 37 °C for 30 min. Fluorescence images of Calcein (a) and CaPF-1 (b) were observed by the excitation at 559 nm and 635 nm, respectively. A merged image (c) was obtained from (a) and (b). Scale bar: 10 $\mu$m.

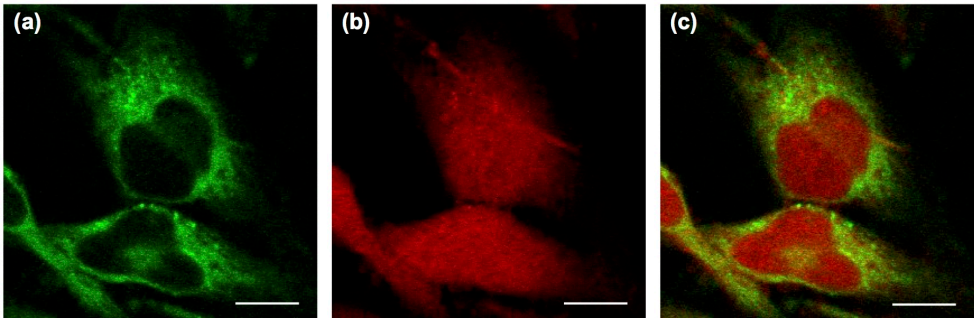


**Fig. S9** Co-staining experiment of HeLa cells with ER Tracker Red and CaPF-1. The cells were loaded with ER Tracker Red (0.1 $\mu$M) and CaPF-1-AM (2 $\mu$M) at 37 °C for 30 min. Fluorescence images of Calcein (a) and CaPF-1 (b) were observed by the excitation at 559 nm and 635 nm, respectively. A merged image (c) was obtained from (a) and (b). Scale bar: 20 $\mu$m.

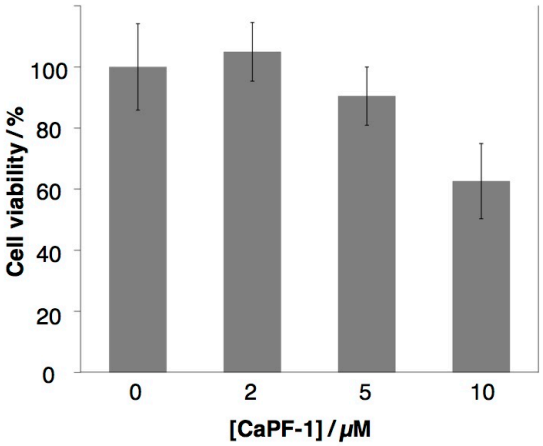


**Fig. S10** Cell viability of CaPF-1-loaded HeLa cells determined by MTT assay. The cells were incubated with CaPF-1-AM (0, 2, 5, and 10 $\mu$M) in HBSS containing 0.4% DMSO and 0.04% Pluronic F-127 in a $CO_2$ incubator for 4 h. The results are expressed as percentages of the dye-free controls. All data are presented as mean standard deviation ($n$ = 16).

**Table S1** Fluorescence quantum yields of CaPF-1 in pH 4.0 and 7.4 buffered-solutions containing 0.5 mM EGTA ($Ca^{2+}$-free) or 1 mM $Ca(NO_3)_2$ ($Ca^{2+}$-bound).

| CaPF-1 | $\Phi_F$ ($Ca^{2+}$-free) | $\Phi_F$ ($Ca^{2+}$-bound) |
|---|---|---|
| pH 4.0 | > 0.01 | > 0.01 |
| pH 7.0 | ~ 0.02 | 0.22 |

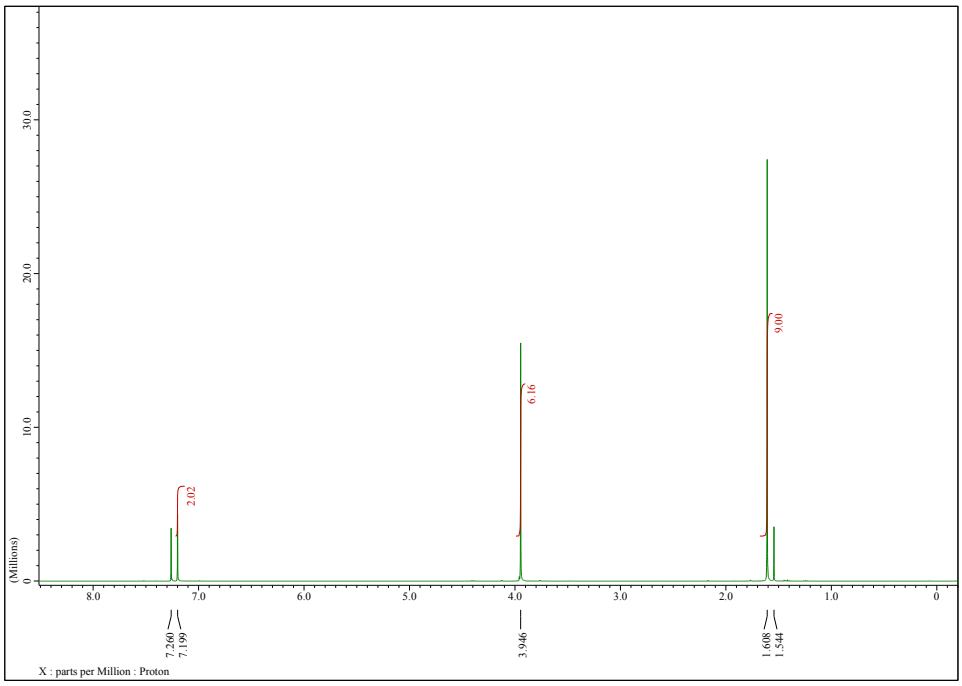


**Fig. S11** $^{1}$H NMR spectrum of *tert*-Butyl 4-bromo-3,5-dimethoxybenzoate (400 MHz, $CDCl_3$).

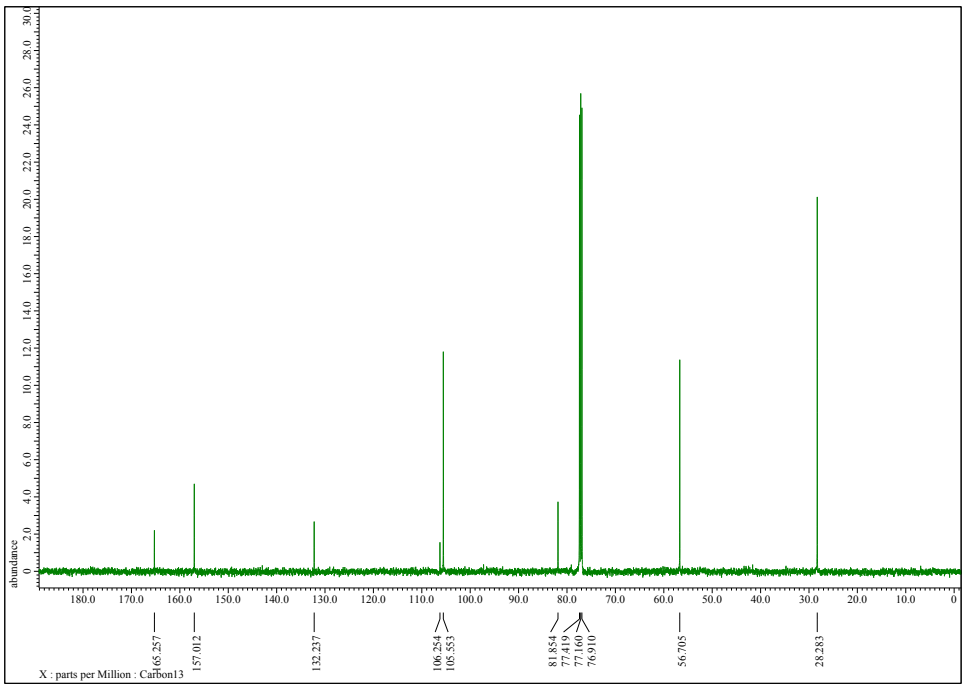


**Fig. S12** $^{13}C\{^{1}H\}$ NMR spectrum of *tert*-Butyl 4-bromo-3,5-dimethoxybenzoate (125 MHz, $CDCl_3$).

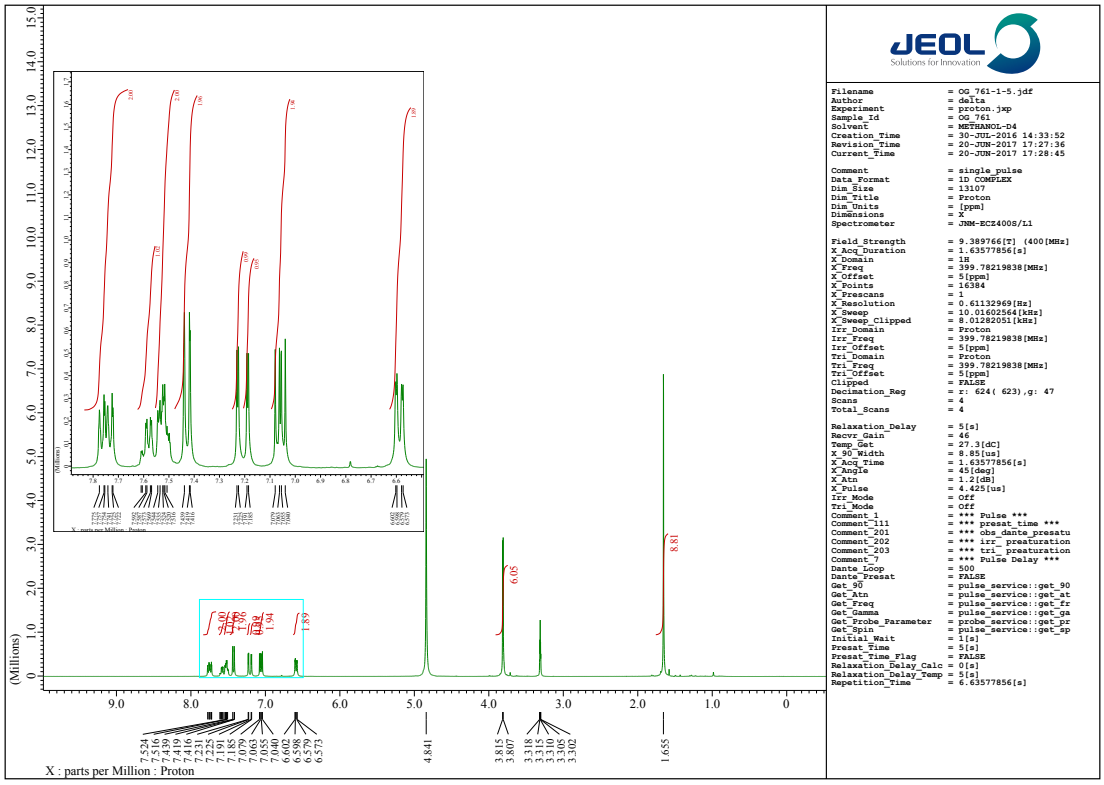


**Fig. S13** $^{1}$H NMR spectrum of **2** (400 MHz, $CD_3OD$).

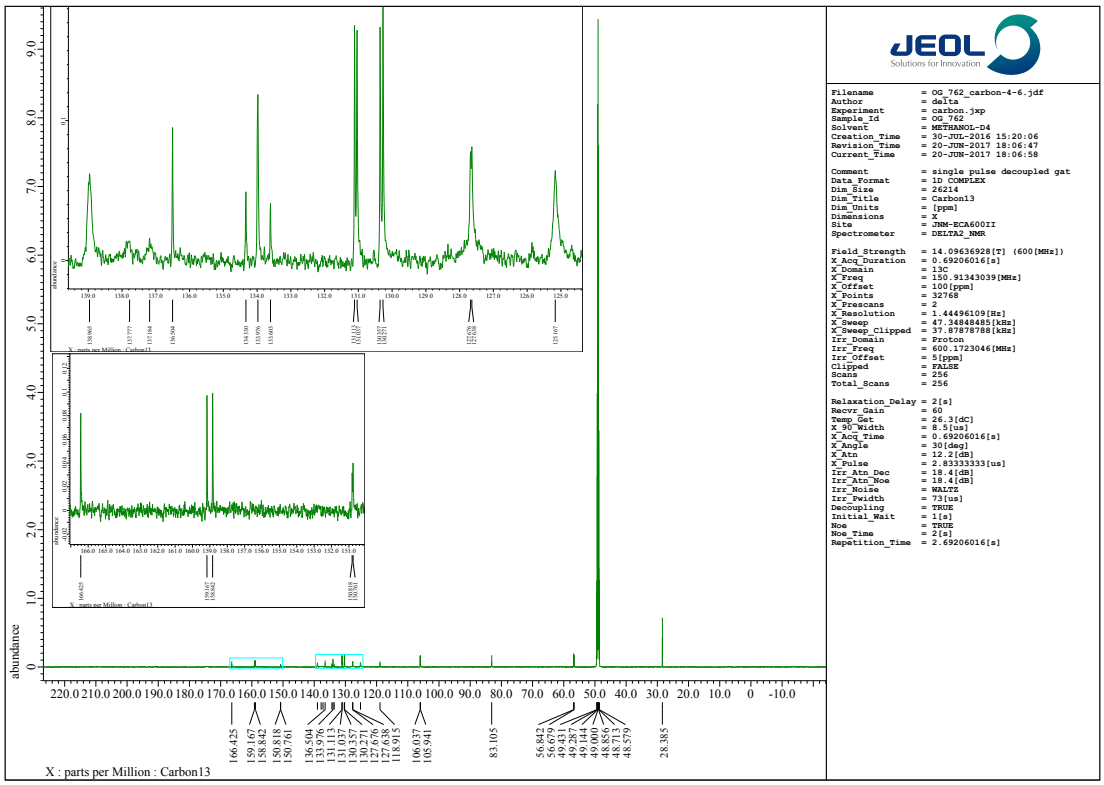


**Fig. S14** $^{13}C\{^{1}H\}$ NMR spectrum of **2** (150 MHz, $CD_3OD$).

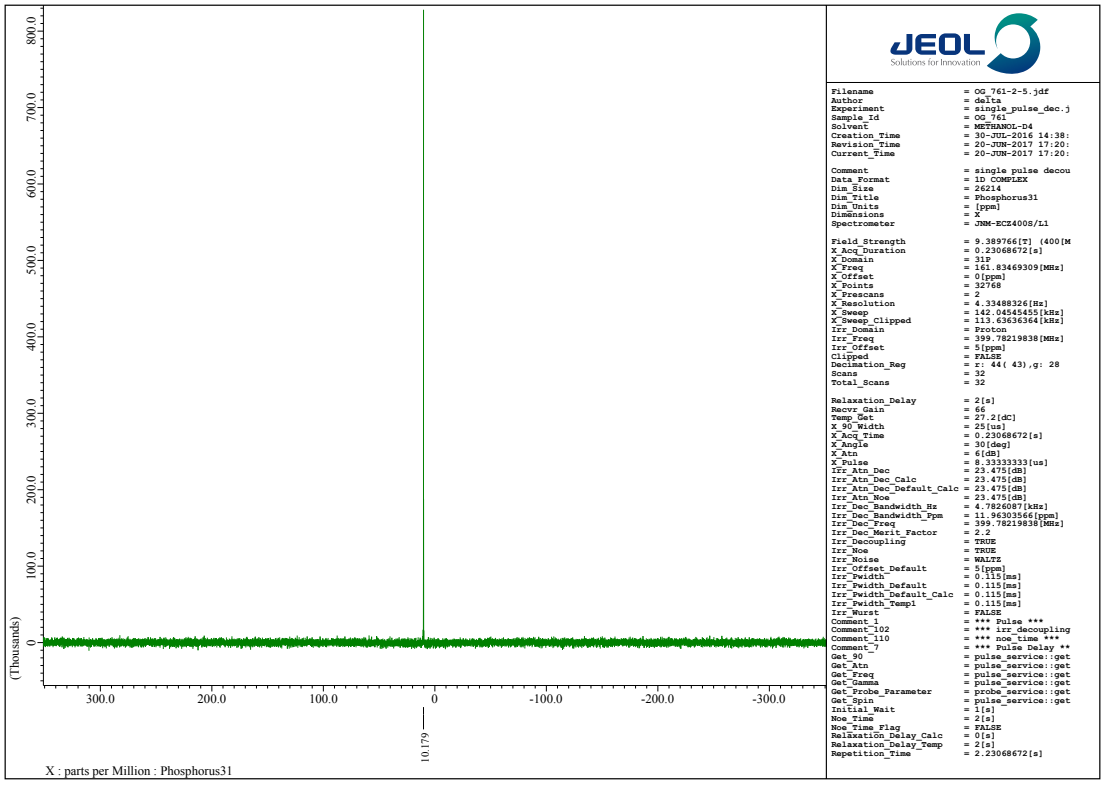


**Fig. S15** $^{31}$P NMR spectrum of **2** (162 MHz, $CD_3OD$).

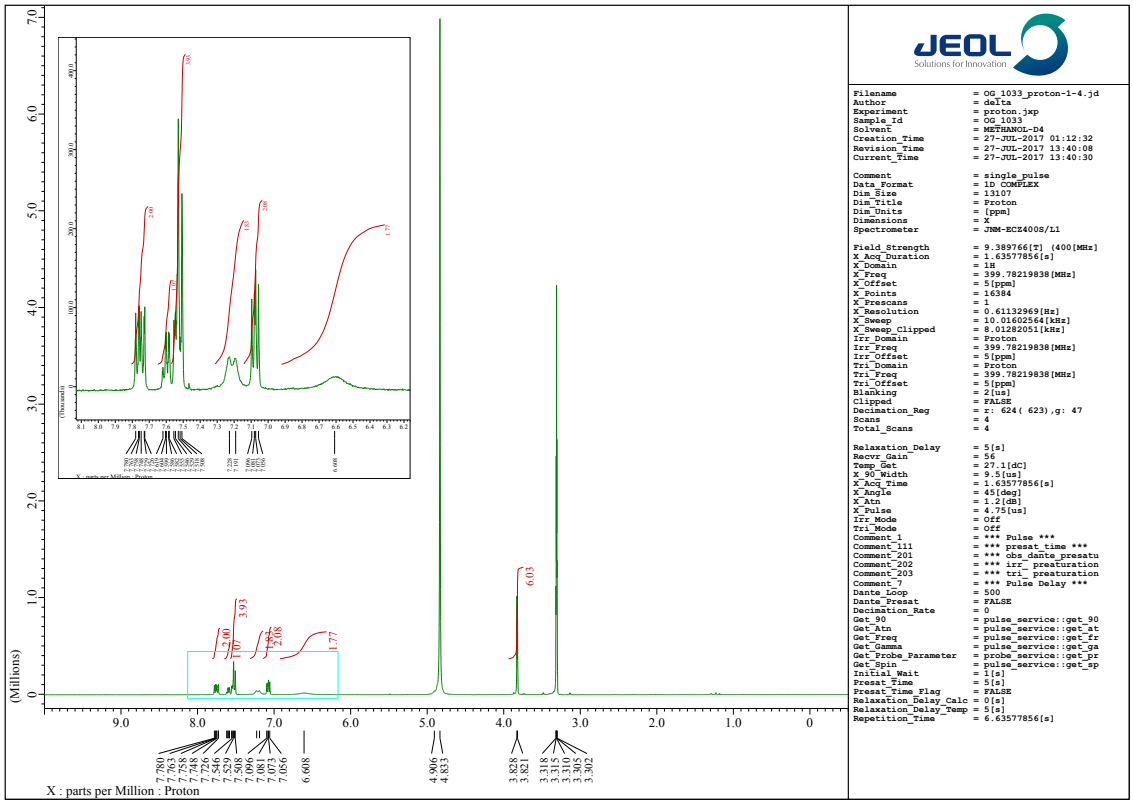


**Fig. S16** $^{1}$H NMR spectrum of **3** (400 MHz, $CD_3OD$).

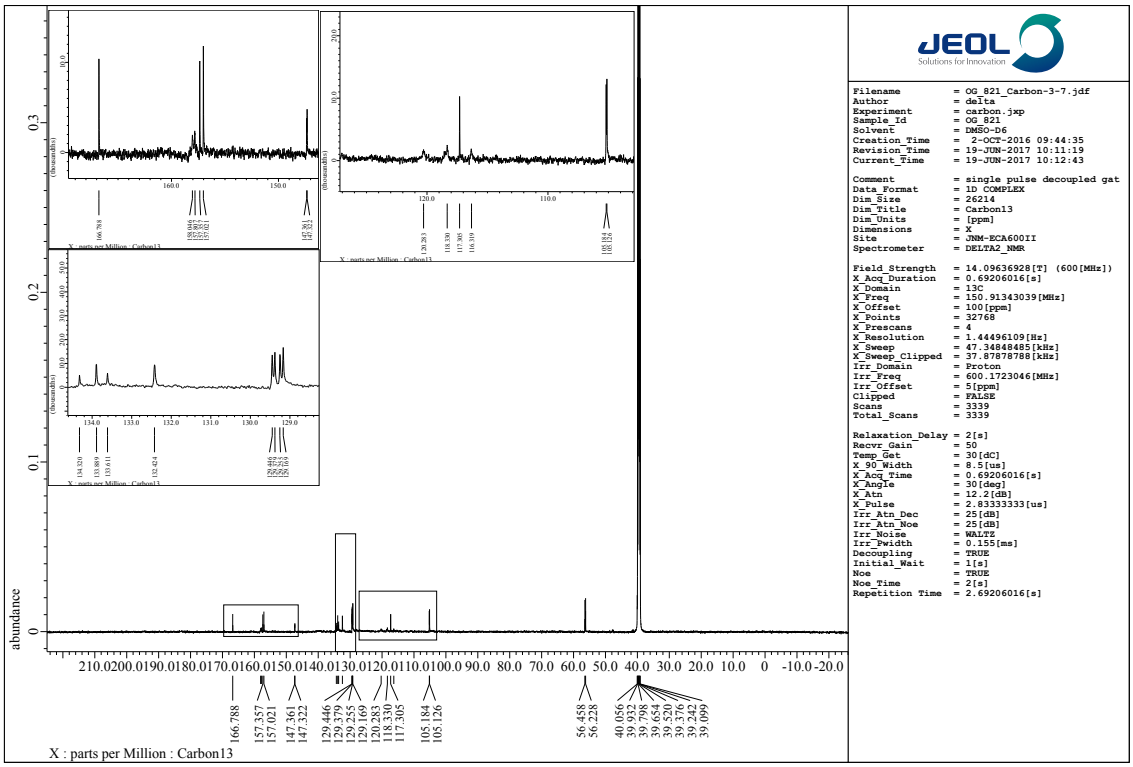


**Fig. S17** $^{13}C\{^{1}H\}$ NMR spectrum of **3** (150 MHz, DMSO-$d_6$).

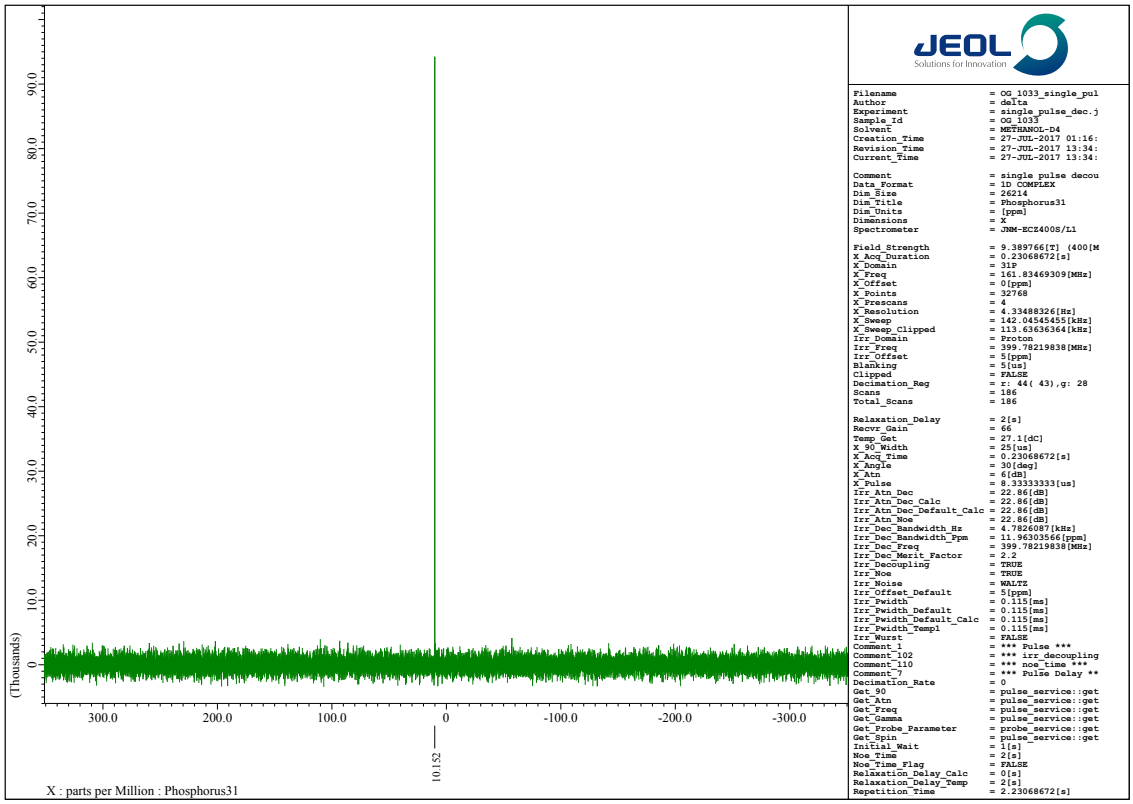


**Fig. S18** $^{31}P$ NMR spectrum of **3** (162 MHz, $CD_3OD$).

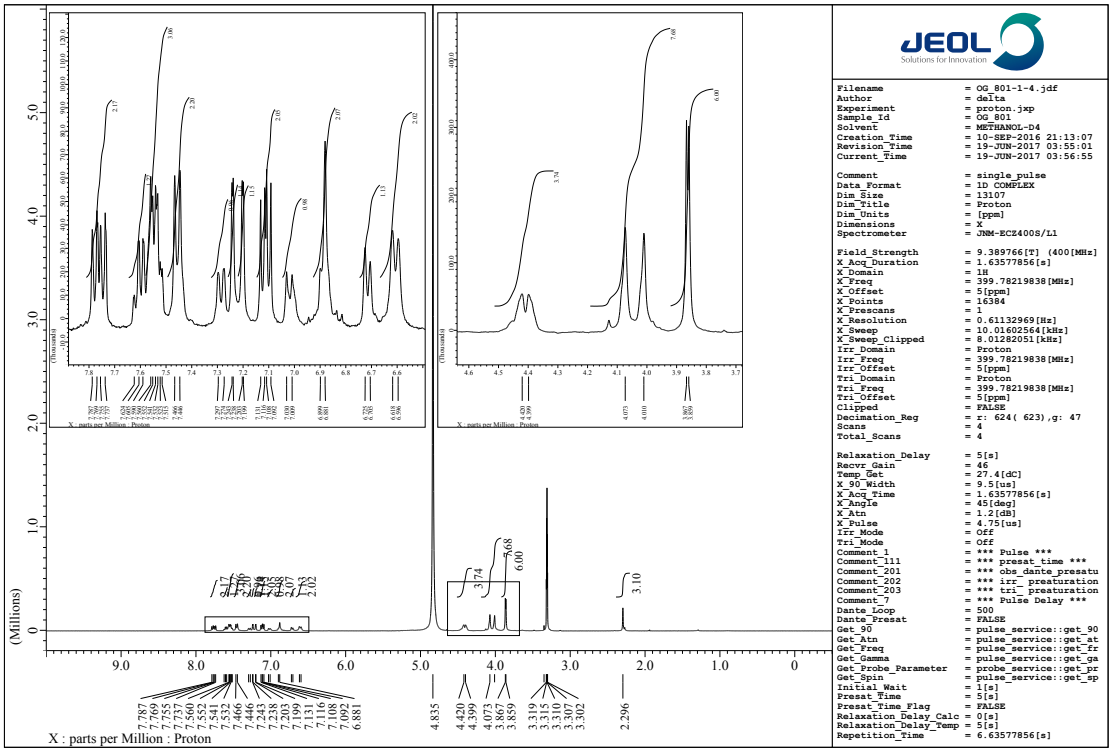


**Fig. S19** $^{1}$H NMR spectrum of CaPF-1 (400 MHz, $CD_3OD$).

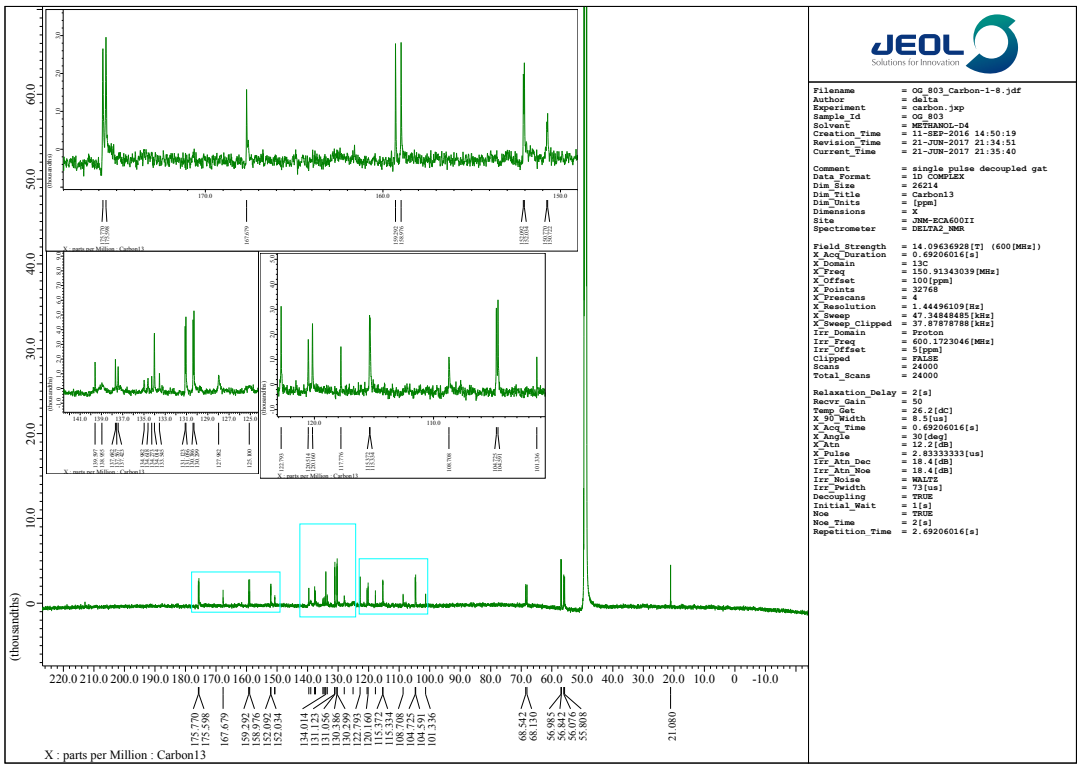


**Fig. S20** $^{13}C\{^{1}H\}$ NMR spectrum of CaPF-1 (150 MHz, $CD_3OD$).

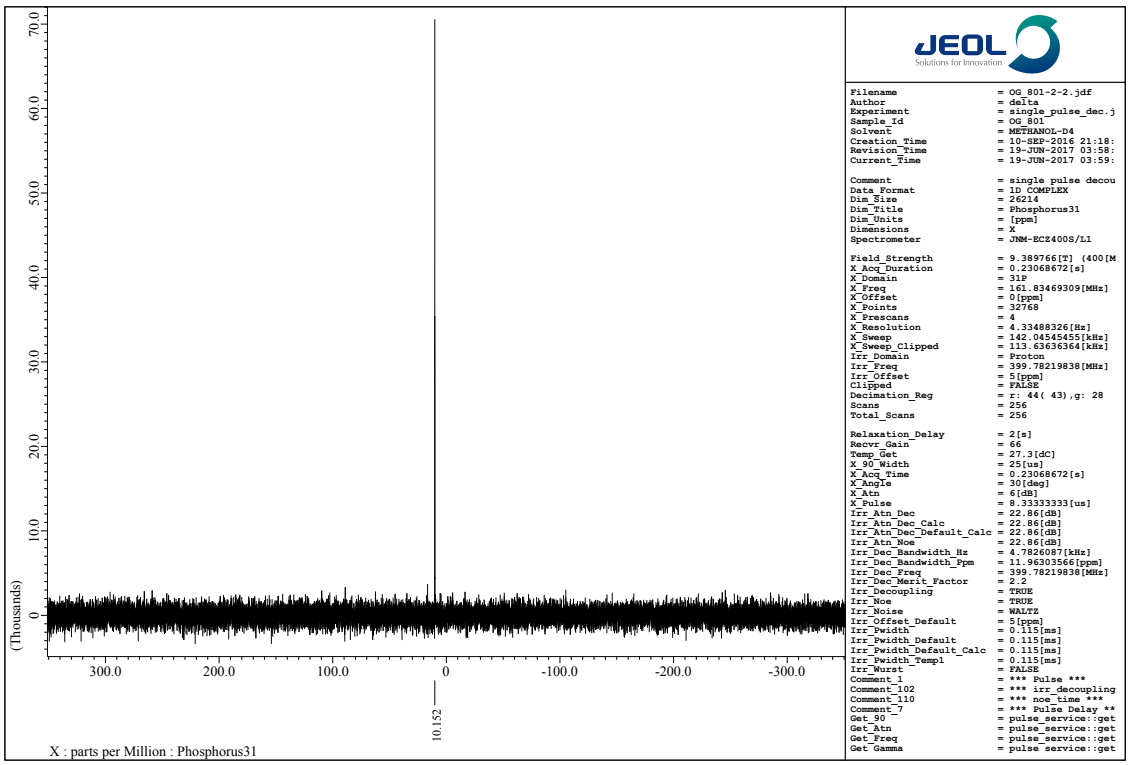


**Fig. S21** $^{31}$P NMR spectrum of CaPF-1 (162 MHz, $CD_3OD$).

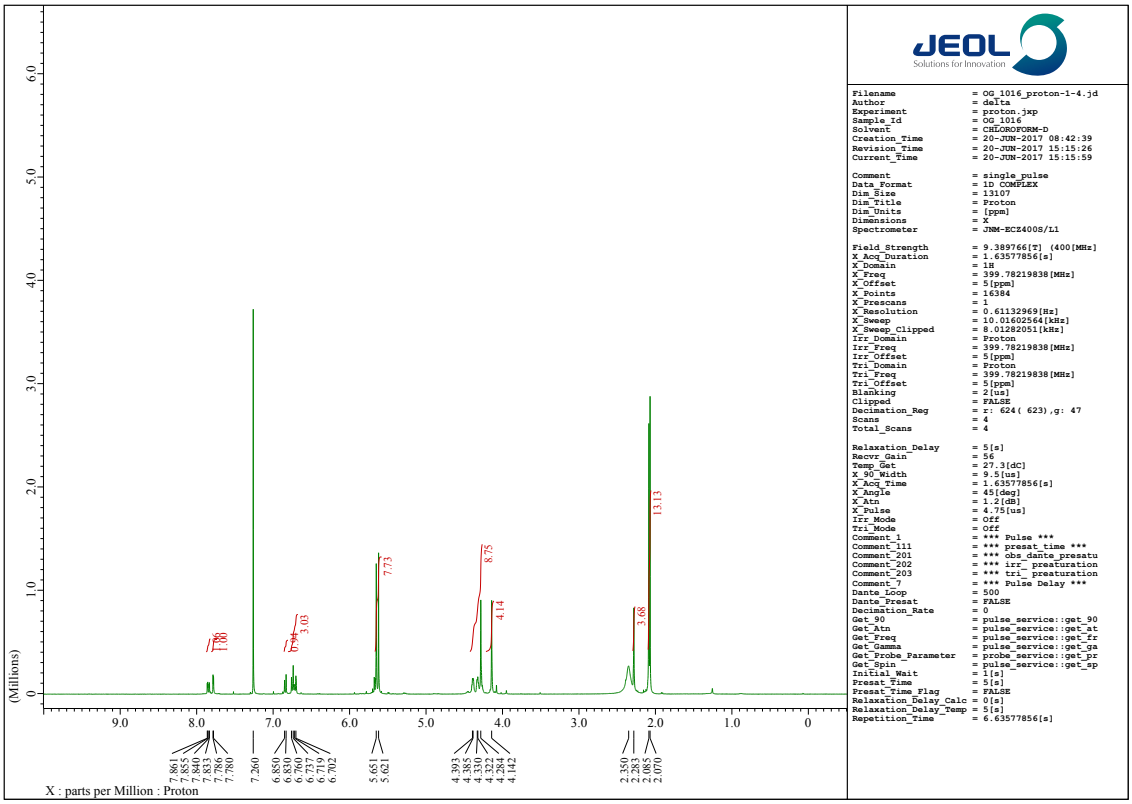


**Fig. S22** $^{1}$H NMR spectrum of **6** (400 MHz, $CDCl_3$).

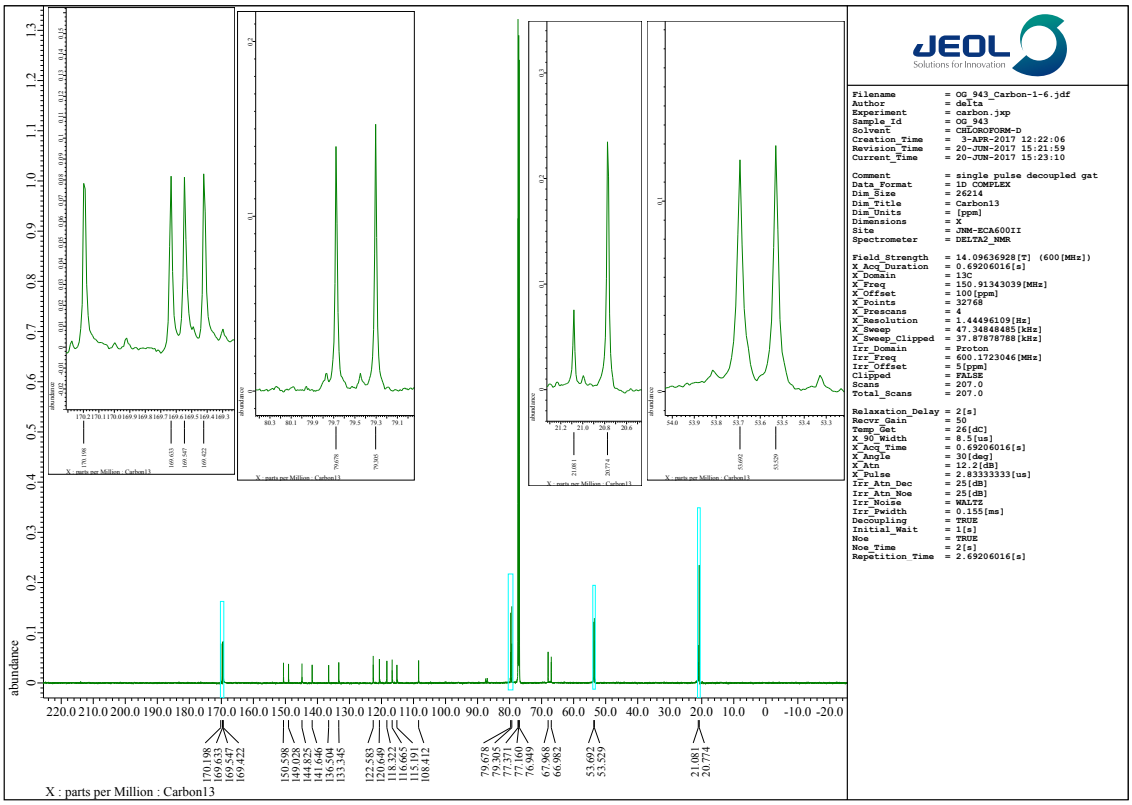


**Fig. S23** $^{13}C\{^{1}H\}$ NMR spectrum of **6** (150 MHz, $CDCl_3$).

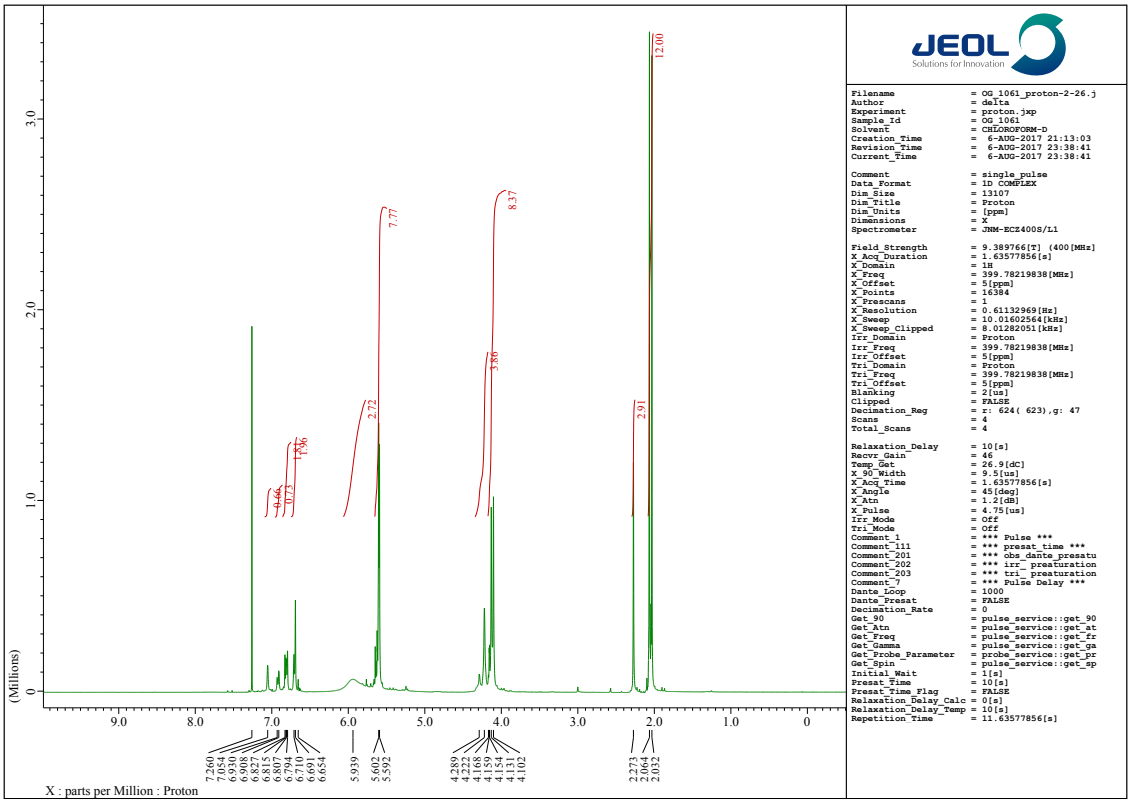


**Fig. S24** $^{1}H$ NMR spectrum of **5** (400 MHz, $CDCl_3$).

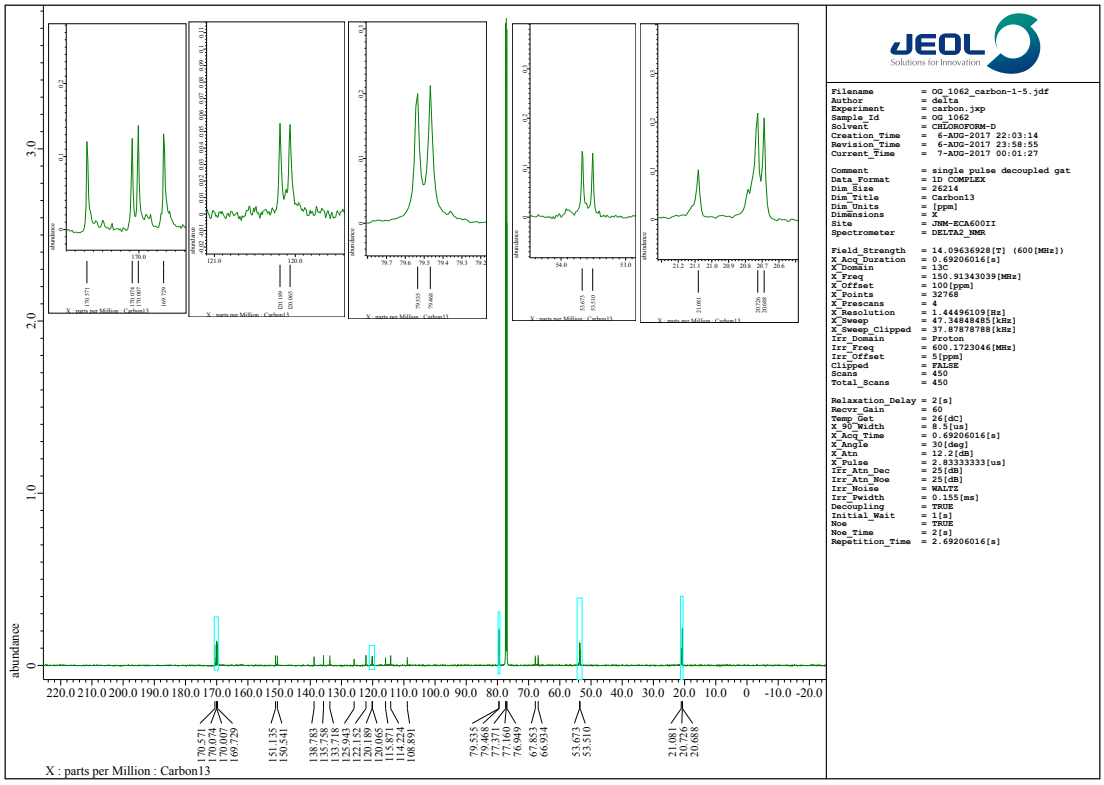


**Fig. S25** $^{13}C\{^{1}H\}$ NMR spectrum of **5** (150 MHz, $CDCl_3$).

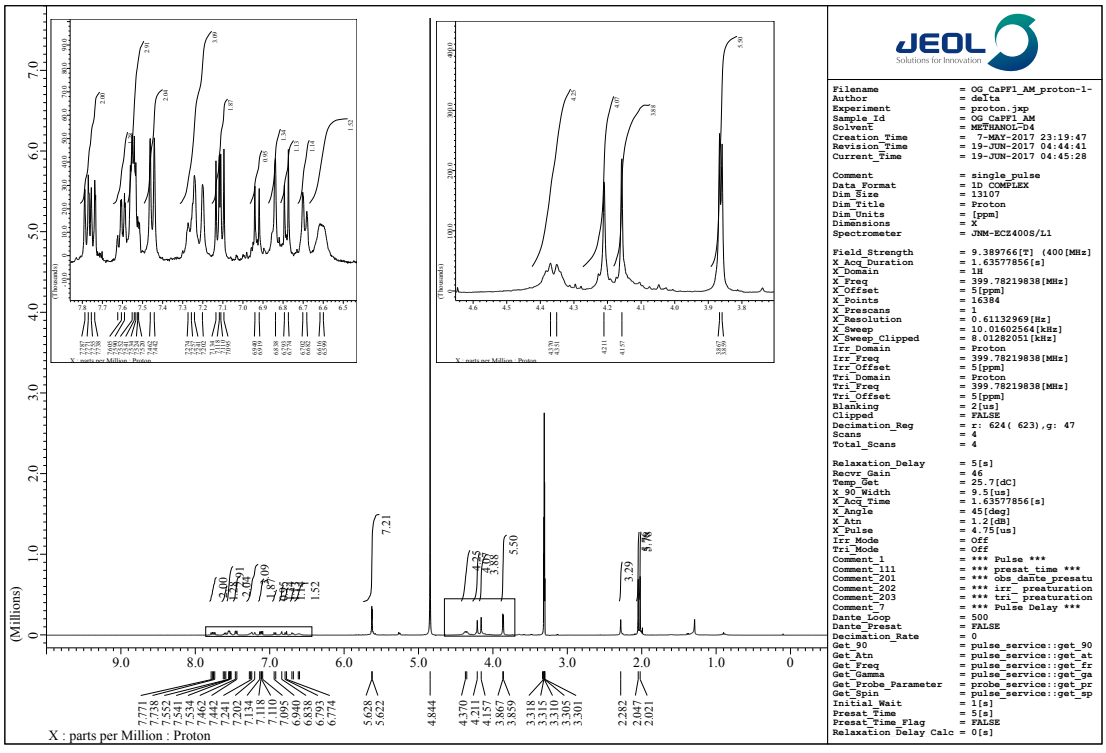


**Fig. S26** $^{1}H$ NMR spectrum of CaPF-1-AM (400 MHz, $CD_3OD$).

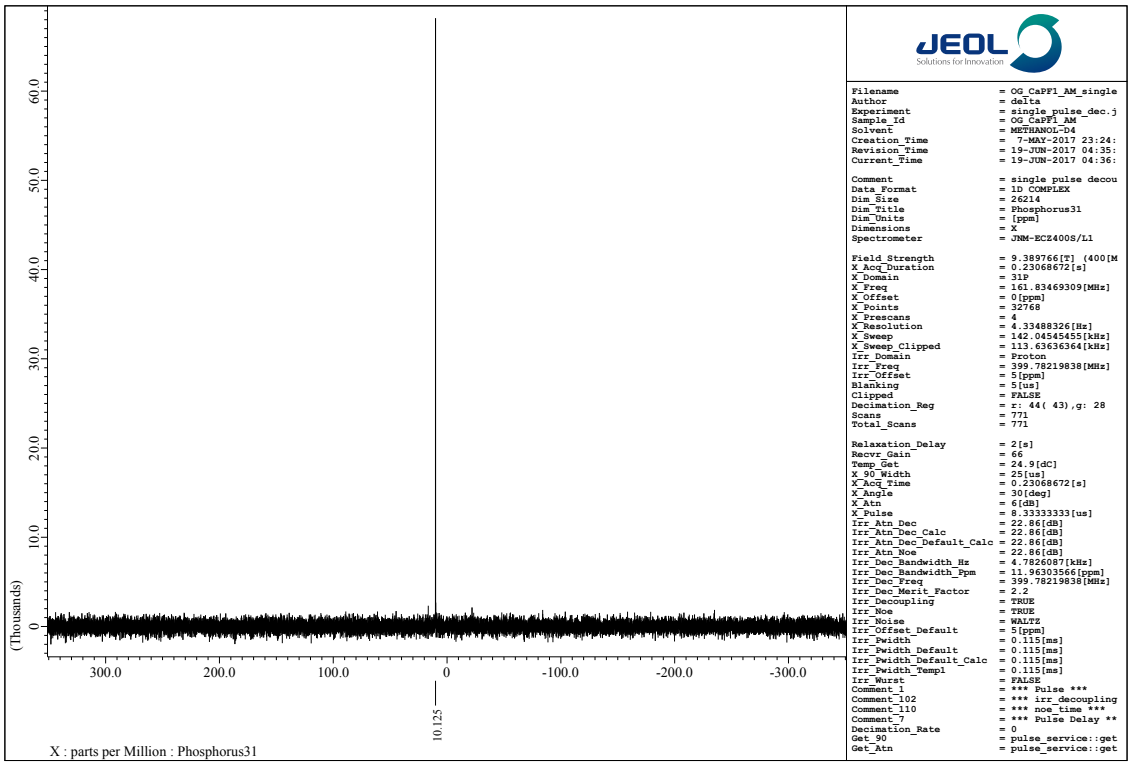


**Fig. S27** $^{31}$P NMR spectrum of CaPF-1-AM (162 MHz, $CD_3OD$).