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Design and Synthesis of a 4-Nitrobromobenzene Derivative Bearing an Ethylene Glycol Tetraacetic Acid Unit for a New Generation of Caged Calcium Compounds with Two-Photon Absorption Properties in the Near-IR Region and Their Application in Vivo

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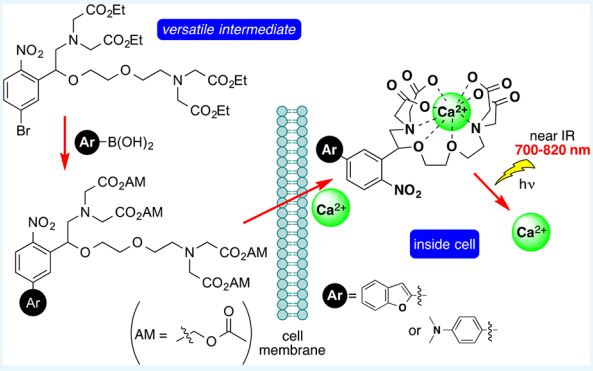
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S *Supporting Information*

ABSTRACT: Among biologically active compounds, calcium ions (Ca^{2+}) are one of the most important species in cell physiological functions. Development of new calcium chelators with two-photon absorption (TPA) properties is a state-of-the-art challenge for chemists. In this study, we report the first and efficient synthesis of 5-bromo-2-nitrobenzyl-substituted ethylene glycol tetraacetic acid (EGTA) as a platform for a new generation of calcium chelators with TPA properties in the near-infrared region. New calcium chelators with high TPA properties, that is, a two-photon (TP) fragmentation efficiency of δ_u = 20.7 GM at 740 nm for 2-(4-nitrophenyl)benzofuran (NPBF)-substituted EGTA (NPBF-EGTA, K_d = 272 nM) and δ_u = 7.8 GM at 800 nm for 4-amino-4′-nitro-1,1′-biphenyl (BP)-substituted EGTA (BP-EGTA, K_d = 440 nM) derivatives, were synthesized using Suzuki−Miyaura coupling reactions of the bromide with benzofuran-2-boronic acid and 4-(dimethylamino)phenyl boronic acid, respectively. The corresponding acetoxymethyl (AM) esters were prepared and successfully applied to the Ca^{2+}-uncaging reaction triggered by TP photolysis in vivo.



INTRODUCTION

Calcium ions (Ca^{2+}) play pivotal roles in various biological phenomena,[1−3] including sending and receiving nerve motions, muscle contractions, delivery of hormones and other chemicals such as neurotransmitters, maintaining a normal heartbeat, and blood clotting. Artificial control of Ca^{2+} levels, [Ca^{2+}], in cells would be key to elucidating the role of Ca^{2+} in biological phenomena. Ca^{2+} levels may be controlled by the "encapsulation (=caging) and release (=uncaging)" of Ca^{2+}. Biologically active cations (Ca^{2+}) can be caged by organic chelators[4−9] and uncaged by photo-triggered structural changes in the chelator.

Since 1980, the structures of ethylene glycol tetraacetic acid (EGTA)[5] and 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA)[10,11] have been known as excellent chelators that bind selectively to Ca^{2+} (Figure 1). Indeed, Ellis-Davies and co-workers used an EGTA chelator for the photochemical uncaging of biologically active Ca^{2+}.[4−9] In 2012, BAPTA was used by Campo and co-workers for pH-independent caging of Ca^{2+} as well as its uncaging by photolysis.[11]

For state-of-the-art physiological experiments in living cells and organisms, two-photon (TP) uncaging process using near-infrared (IR) light is becoming essential.[4−9,12−25] Alternatively, lanthanide-doped upconversion nanoparticles have also proved to be efficient in recent years.[26−32] Meanwhile, the three-dimensional (3D) resolution achieved using TP excitation is higher than that of one-photon (OP) excitation reactions. Furthermore, the near-IR TP excitation process allows deeper

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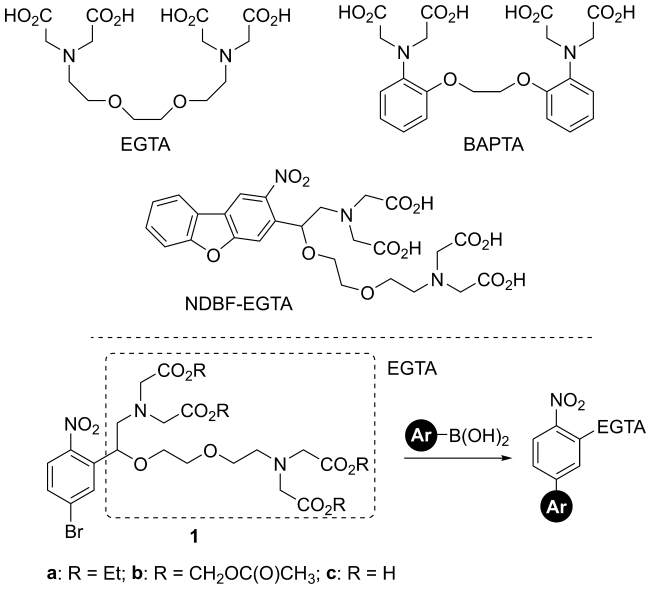


Figure 1. Design and synthesis of new TPA chromophores bearing EGTA unit from platform **1a**.

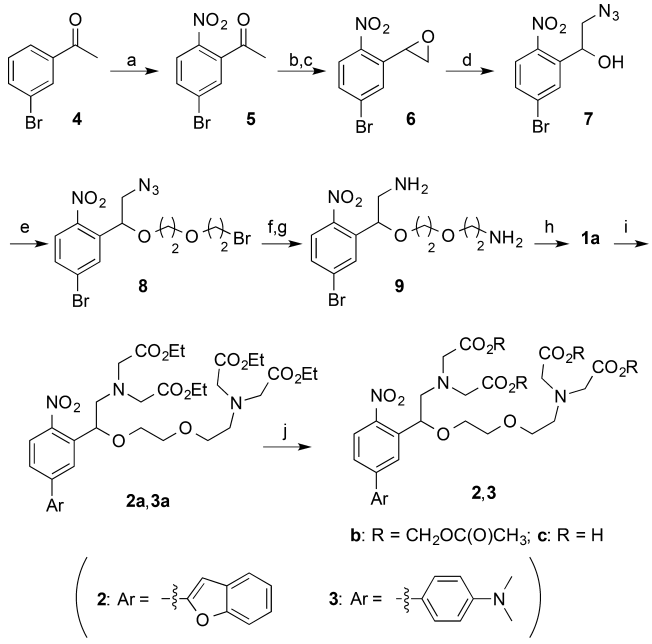
penetration of the light beam in living tissue and reduced light scattering.[4−9,12−25] The optical absorption by endogenous chromophores is also reduced, so less photo-damage to cells is expected. To date, nitrodibenzofuran (NDBF)-substituted EGTA (NDBF-EGTA)[5] is the only Ca^{2+} chelator reported to have TP uncaging reactivity when excited at 720 nm (Figure 1). The TP uncaging cross section (δ_u), $\delta_u = \sigma_2 \times \varphi$, was reported to be ∼0.6 Göppert-Mayer (GM)[33] (1 GM = 10^{-50} cm^4 s/photon/molecule). The value of σ_2 is the two-photon absorption (TPA) cross section in GM, and φ is the quantum yield of the uncaging reaction. High cross-section GM values in the TP uncaging reaction, for example, 3 GM,[34] have been suggested as a requirement for physiological studies.

In the present study, we report the first and efficient synthesis of 5-bromo-2-nitrobenzyl-substituted EGTA derivative **1a** (R = Et), which is a versatile platform for a new generation of caged calcium compounds (Figure 1). Bromide **1a** can be utilized for the synthesis of a wide variety of TP-responsive Ca^{2+} chelators, using Suzuki−Miyaura coupling reactions.[35] Thus, the TP absorption maximum and TP reactivity can be tuned by selecting a different aryl group (Ar) in boronic acids $ArB(OH)_2$. Compounds **2** and **3** (Scheme 1) were prepared as examples in this study. Efficiencies of TP-induced bond cleavage reactions were found to be as high as δ_u = 20.7 GM at 740 nm for **2a** and δ_u = 7.8 GM at 800 nm for **3a**. The corresponding acetoxymethyl (AM) esters **2b** and **3b** were synthesized and were shown to be effective for in vivo Ca^{2+} uncaging reaction.

RESULTS AND DISCUSSION

Synthesis of Compounds 1a, 2, and 3. The synthesis of **1a** is summarized in Scheme 1. Commercially available starting material 3-bromo-acetophenone (**4**) was nitrated[36] with nitric acid and sulfuric acid to afford nitro-substituted compound **5** in 64% yield. Bromination of **5** with Br_2 in the presence of acetic acid in dichloromethane at room temperature (RT) provided the brominated compound in 99% yield. Without further purification, the keto function of the brominated compound obtained was reduced using sodium borohydride, followed by

Scheme 1. Synthesis of 1a, 2, and 3[a]



[a]Reagents and conditions: (a) KNO_3, H_2SO_4, CH_2Cl_2, 0 °C−RT, 4 h, 64%; (b) Br_2, $AcOH/CH_2Cl_2$, RT, 55 h, 99%; (c) $NaBH_4$, aq NaOH (2.5 M), 1,4-dioxane/MeOH, 0 °C, 1 h, 71%; (d) NaN_3, DMF, RT−40 °C, 4 h, 40%; (e) bis(2-bromoethyl) ether, Cs_2CO_3, ACN, RT−50 °C, 5 h, 53%; (f) NaN_3, DMF, RT, 16 h, 80%; (g) PPh_3, RT, 24 h, 80%; (h) NaI, DIEPA, ethyl bromoacetate, ACN, 0−80 °C, 3 h, 70%, (i) $ArB(OH)_2$, $Pd(PPh_3)_4$, K_2CO_3, THF, H_2O, RT−60 °C, 3 h, 95% for **2a** and 90% for **3a**; (j) (I) KOH, MeOH, 0 °C−RT, 16 h; (II) bromomethyl acetate, DIPEA, DMF, 0 °C−RT, 16 h, 60%. DIPEA = *N*,*N*-diisopropylethylamine; THF = tetrahydrofuran; ACN = acetonitrile.

in situ cyclization under alkaline treatment for 30 min at 0 °C, giving oxirane **6** in 71% yield. Epoxide **6** was subsequently treated with sodium azide[37] in dimethylformamide (DMF) at 40 °C to afford hydroxyl azide **7** in 40% yield. This step was the key in our construction of the EGTA unit. The reaction yield was largely dependent on the equivalents of sodium azide and the reaction temperature. After several attempts, 1 equiv NaN_3 and ∼40 °C were found to be the best reaction conditions. Hydroxyl azide was alkylated[38] with bis(2-bromomethyl)ether in the presence of cesium carbonate in DMF at 50 °C to give compound **8** in 53% yield. The isolated alkylated compound **8** was treated with sodium azide at RT to give the diazide[39] in 80% yield. The diazide was then converted to diamine **9** in 80% yield by treatment with PPh_3,[40] followed by hydrolysis. Diamine **9** was alkylated[41] with ethyl bromoacetate in the presence of sodium iodide in CH_3CN to give tetraacetic acid derivative **1a** in 70% yield.

Bromide **1a** is a versatile intermediate in Suzuki−Miyaura coupling reactions for producing a new generation of Ca^{2+} chelators with TPA properties. Numerous TPA chromophores can be prepared from the **1a** platform. For example, in this study, bromide **1a** was treated with benzofuran-2-boronic acid or 4-(dimethylamino)phenylboronic acid in the presence of $Pd(PPh_3)_4$ and K_2CO_3 to afford EGTA derivatives **2a** or **3a** with benzofuran or biphenyl units as TP chromophores in 95% and 90% yield, respectively. Ethyl esters **2a** and **3a** were hydrolyzed with potassium hydroxide (KOH), followed by esterification with acetoxy methyl (AM) ester to give **2b** and **3b**

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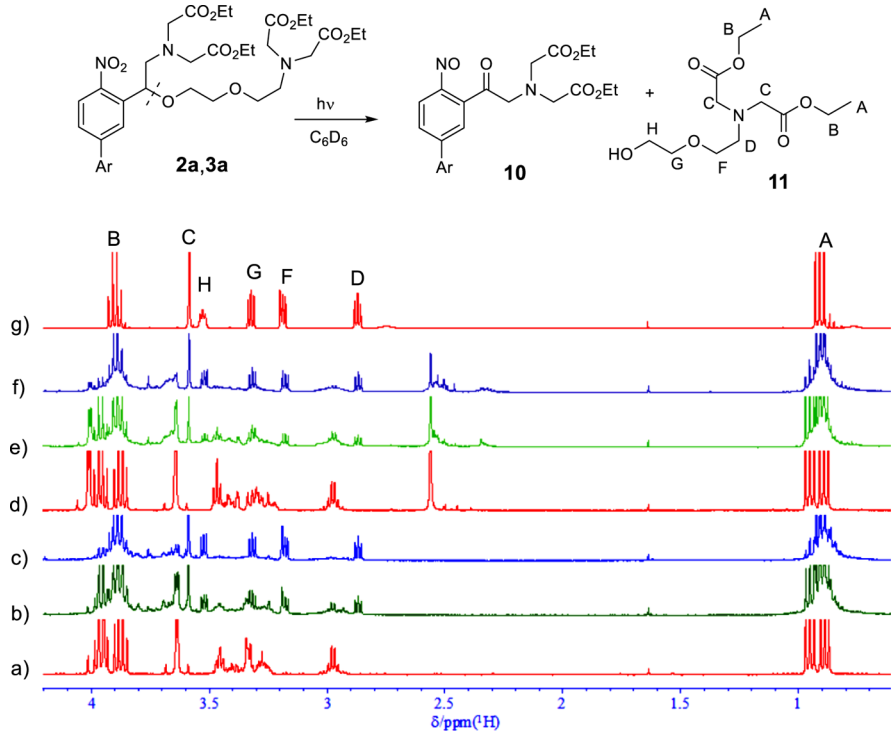


Figure 2. ^{1}H NMR (400 MHz), (a) δ 0.5−4.2 ppm of **2a** in C_6D_6, (b) after 4 h, and (c) 8 h irradiation at 360 nm; (d) ^{1}H NMR (400 MHz), δ 0.5−4.2 ppm of **3a** in C_6D_6, (e) after 5 h, and (f) 10 h irradiation at 400 nm; and (g) ^{1}H NMR (400 MHz), δ 0.5−4.2 ppm of **11** in C_6D_6.

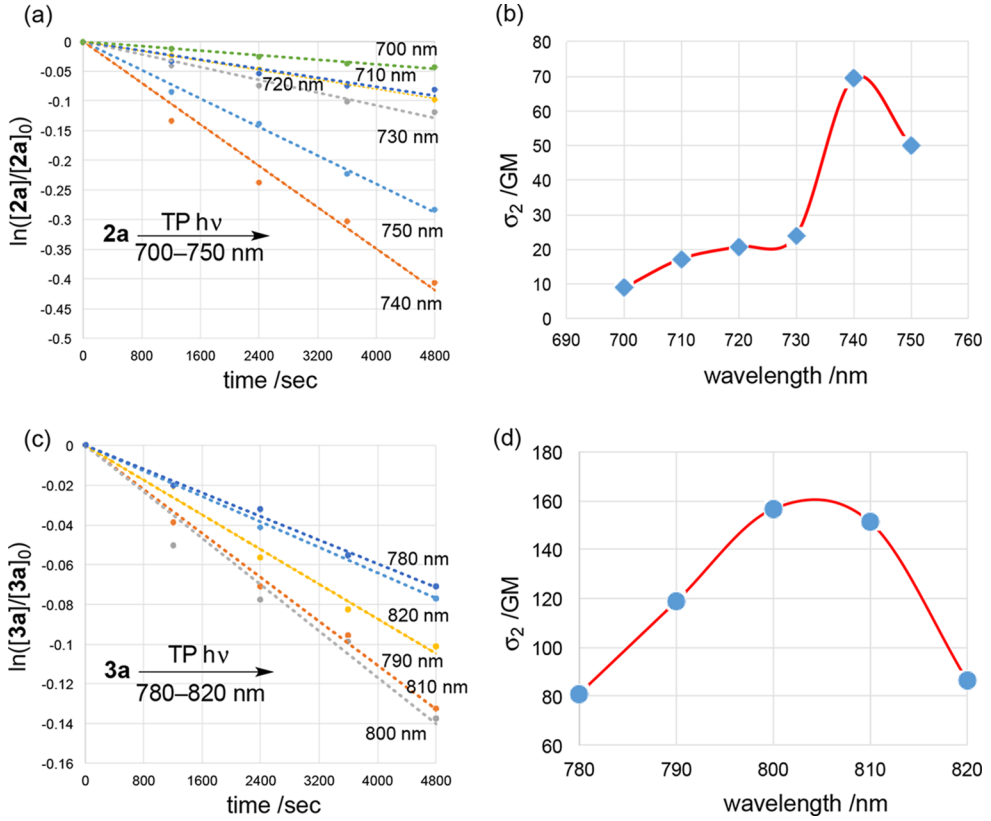


Figure 3. (a) Time profile of TP uncaging of **2a** ln([**2a**]/[**2a**]$_0$) vs irradiation time at 700−750 nm, (b) an action TP absorption spectrum of **2a**, (c) time profile of TP uncaging of **3a** ln([**3a**]/[**3a**]$_0$) vs irradiation time at 780−820 nm, and (d) an action TP absorption spectrum of **3a**.

in 60% yield. AM ester **2b** was loaded into cells to generate intracellular EGTA calcium chelators, vide infra.

OP Photolysis of 2a and 3a. The photoreactions of compounds **2a**[42] and **3a** were investigated first using an OP excitation process (Figure 2). The OP photochemical reaction

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of **2a** (10 mM in C_6D_6, λ_{max} = 362 nm with ε 18 771 M^{-1} cm^{-1}) was conducted at 360 ± 10 nm using a monochromator with a xenon lamp (500 W). The photo-induced bond cleavage reaction was monitored using ^{1}H NMR (400 MHz) spectroscopic analysis (Figure 2a–c). As shown in our preliminary results on the photolysis of **2a**,[42] clear decomposition of **2a** was confirmed after irradiation for 8 h. Using ^{1}H NMR analysis, fragmentation product **11** was observed in the photolysate. Compound **11** was prepared in this study to confirm its formation during the photolysis. The quantum yield (φ) of the photochemical decomposition of EGTA ethyl ester **2a** was determined to be 0.3 ± 0.05 at 360 nm irradiation using a xenon lamp. Ferric oxalate was used as a chemical actinometer to determine the quantum yield.[43]

The OP bond cleavage reaction of **3a** (10 mM in C_6D_6, λ_{max} = 403 nm with ε 8790 M^{-1} cm^{-1}) was also conducted at 400 ± 10 nm using a xenon lamp (Figure 2d–f). The photo-induced bond cleavage was monitored using ^{1}H NMR (400 MHz) analysis. The complete structural decomposition of compound **3a** was observed after irradiation for 10 h. The quantum yield of the decomposition of EGTA ethyl ester **3a** was determined to be 0.05 at 400 nm irradiation.

TP Photolysis of 2a and 3a. Photochemical reactions using TP excitation by near-IR light were conducted for **2a** and **3a** in dimethyl sulfoxide (DMSO). The TP photolysis of **2a** was performed at 700, 710, 720, 730, 740, and 750 nm with a Ti:sapphire laser using ~700 mW at ~25 °C (Figure 3a,b). The TP photolysis of **3a** was conducted at 780, 790, 800, 810, and 820 nm at ~25 °C (Figure 3c,d). Using high-performance liquid chromatography (HPLC) analysis, TP-induced bond cleavage reactions were monitored by the consumption of **2a** or **3a**. Wavelength-dependent changes in the rate constants were determined by the TP decomposition of **2a** or **3a**. As reported previously, the absolute value of the TPA cross section for 2-(4-nitrophenyl) benzofuran was 18 GM at 720 nm.[42] The TP action spectra for **2a** and **3a** were extrapolated in comparison with the standard value. Thus, the TP absorption characters of **2a** and **3a** were determined to be ~70 GM at 740 nm and ~160 GM at 800 nm, respectively (Figure 3). The TP efficiencies obtained for photochemical decomposition were 20.7 GM at 740 nm for **2a** and 7.8 GM at 800 nm for **3a**.

Measurement of Ca^{2+} Binding Affinities. The dissociation constants, K_d, of Ca^{2+} ions with **2c** and **3c** (R = H) were determined by a titration method using fluo-3 fluorescent dye[44,45] (Figures 5 and 6). Thus, the concentration of free Ca^{2+} ions, $[Ca^{2+}]_F$, after treating 1.0 mM solutions of **2c** and **3c** was determined by fluorescence intensity at 530 nm from fluo-3 (for a detailed procedure, see Experimental Section). The Scatchard analyses shown in Figures 5 and 6 provided dissociation constants of K_d = 272 nM for **2c** and K_d = 440 nM for **3c**.

Physiologic Studies of 2b In Vivo. Because compound **2a** had a TP efficiency larger than that of **3a**, we loaded cultured neurons with AM ester **2b** (100 μM, 35 °C for 40 min) to stimulate presynaptic terminals with TP uncaging. We recorded inhibitory postsynaptic currents (IPSCs) from whole-cell-clamped neurons by irradiating with a 720 nm-wavelength femtosecond laser (140 fs, 7.6 mW, 1 ms) at 100 points, encompassing a small dendritic region (Figure 4a). The mapping experiments were repeated 81 times in four cells. We recorded IPSCs selectively at dendrite edges, where synapses are most frequently formed. Laser irradiation seldom induced currents over numerous uncaging events on the dendrites (Figure 4b), supporting that the IPSCs are not caused by damages of the plasma membrane in the presynaptic terminals. The time course and amplitude of the uncaging-induced IPSCs were similar to those of the spontaneous IPSCs, except that they were elicited shortly after uncaging (Figure 4b,c). The mean latency of 2.5 ms (±0.4 ms, n = 150) was similar to those of IPSCs evoked by electrical stimulation. Uncaging-induced IPSCs were recorded in only up to four out of 81 sequences applied to the dendrite, indicating that the increase in $[Ca^{2+}]_i$ was smaller than that induced by action potentials. Thus, we have succeeded in stimulating presynaptic terminals directly by TP uncaging to elicit neurotransmitter release, whereas further improvements to the TP excitation cross section are necessary to make the most of presynaptic terminal stimulation without the action potential.

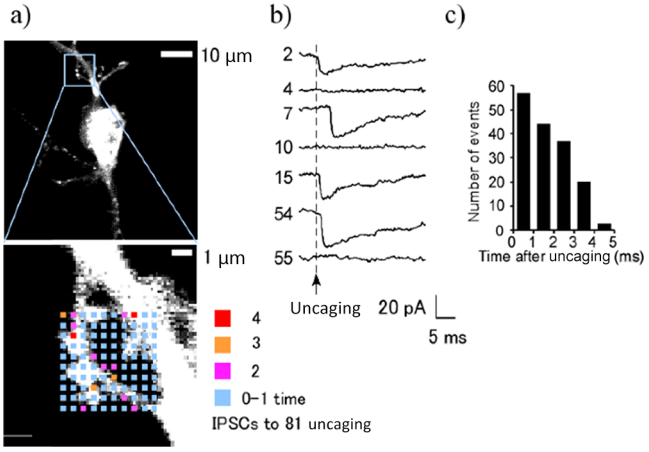


Figure 4. (a) Fluorescence image of a dissociated cultured neuron from cerebral cortices of mice loaded with compound **2b** and a whole cell clamped with Alexa 594 solution. Color squares in the small dendritic region shown in the lower panel indicate the 100 uncaging points separated by 0.55 μm for TP uncaging and mapping of the IPSCs. The mapping was repeated 81 times. The IPSCs from each point were recorded 0–4 times out of 81 sequences, as denoted by color. (b) IPSCs recorded from the single uncaging point of the cell where four IPSCs were recorded. The sequence numbers are shown next to the current traces. (c) Latency histogram for the onset of all uncaging-induced IPSCs recorded from four cells.

CONCLUSIONS

In this study, we designed and synthesized 5-bromo-2-nitrobenzyl-substituted EGTA as a platform for a new generation of calcium chelators with TPA properties. The efficient synthesis of this new bromide was accomplished in nine steps from a commercially available starting material. New calcium chelators with TPA properties, that is, TP fragmentation efficiencies of δ_u = 20.7 GM at 740 nm for NPBF-EGTA (K_d = 272 nM) and δ_u = 7.8 GM at 800 nm for BP-EGTA (K_d = 440 nM) derivatives, were synthesized using a Suzuki–Miyaura reaction to couple the bromide with benzofuran-2-boronic acid and 4-(dimethylamino)phenyl boronic acid, respectively. The corresponding AM esters were synthesized and successfully applied to a Ca^{2+}-uncaging reaction triggered by photolysis in vivo.

EXPERIMENTAL SECTION

All of the reagents and solvents were reagent grade and used without further purification. Thin-layer chromatography (TLC)

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analysis was performed on silica gel plates and imaged under ultraviolet light. ^{1}H NMR and ^{13}C NMR data were recorded with a 400 MHz NMR spectrometer. CD_3OD and $CDCl_3$ [0.03% trimethylsilane (TMS)] were used as deuterated solvents. Chemical shifts are described in parts per million (ppm) relative to TMS (δ = 0.00 ppm), and the coupling constants (J) are stated in hertz (Hz). High-resolution mass spectroscopy (HRMS) analyses were conducted using an Orbitrap XL instrument in the positive ion mode.

The syntheses of **1a**, **2a**, **3a**, **2b**, and **3b** are described below.

1-(5-Bromo-2-nitrophenyl)ethan-1-one (5). A flask was charged with KNO_3 (0.28 g, 2.76 mmol) and cooled to 0 °C, to which conc. H_2SO_4 (5 mL) was added slowly, and the mixture was stirred for 10 min. 3-Bromoacetophenone (**4**) (0.5 g, 2.51 mmol) in CH_2Cl_2 (5 mL) was added at 0 °C. The temperature was raised to the RT, and the mixture was stirred for 4 h. Subsequently, the reaction mixture (RM) was poured into crushed ice. An aqueous layer was extracted with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4, and purified using silica gel column chromatography to give the required nitro product as a white color solid (400 mg, 64%). mp 96−98 °C. IR ν 1710, 1558, 1521, 1345, 1308, 1241, 1087, 900, 858, 830, 756, 694 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.02 (d, J = 8.7 Hz, 1H), 7.75 (dd, J = 8.7 and 2.1 Hz, 1H), 7.56 (d, J = 2.1 Hz, 1H), 2.57 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 198.20, 144.31, 139.43, 133.60, 130.34, 129.52, 125.93, 30.21. HRMS-ESI calcd for $C_8H_6BrNO_3Na$ [M + Na]$^+$ 265.94233, found 265.94223.

2-Bromo-1-(5-bromo-2-nitrophenyl)ethan-1-one (S1). To a solution of **5** (3.7 g, 15.16 mmol) in AcOH−CH_2Cl_2 (1:2, 240 mL) was added bromine (2.42 g, 15.2 mmol), and the RM was stirred at RT for 55 h. The solution was evaporated to give **S1** (4.89 g, 99%). mp 100−103 °C. IR ν 1720, 1560, 1540, 1520, 1347, 1185, 861, 831, 701 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.12 (d, J = 8.8 Hz, 1H), 7.81 (dd, J = 8.8 and 2.1 Hz, 1H), 7.65 (d, J = 2.1 Hz, 1H), 4.3 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 192.82, 144.07, 136.36, 134.31, 132.11, 130.33, 125.81, 33.46. MS-ESI 321.20.

2-(5-Bromo-2-nitrophenyl) Oxirane (6). To a solution of **S1** (1.3 g, 4.02 mmol) in dioxane−MeOH (3:1, 40 mL) was added $NaBH_4$ (156 mg, 4.02 mmol) portionwise at 0 °C, and the RM was stirred for 30 min at the same temperature. The mixture was treated with aqueous NaOH (25 mL, 2.5 mol/L) and stirred for 30 min at 0 °C and then evaporated. The residue was diluted with CH_2Cl_2 (40 mL), washed with water and brine solution, dried over Na_2SO_4, and evaporated. Flash column chromatography (hexane/EtOAc = 10:1) gave a white color solid **6** (710 mg, 71%). mp 72−75 °C. IR ν 1605, 1565, 1524, 1514, 1187, 1097, 990, 898, 864, 835, 756, 699 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.06 (d, J = 8.7 Hz, 1H), 7.8 (d, J = 2.2 Hz, 1H), 7.63 (dd, J = 8.7 and 2.2 Hz, 1H), 4.5 (dd, J = 4.2 and 2.5 Hz, 1H), 3.33 (dd, J = 5.3 and 4.4 Hz, 1H), 2.7 (dd, J = 5.4 and 2.5 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 146.48, 136.66, 131.83, 130.25, 129.84, 126.27, 50.68, 50.34. HRMS-APCI calcd for $C_8H_7{}^{79}BrNO_3Na$ [M + H]$^+$ 243.96038, found 243.96060.

2-Azido-1-(5-bromo-2-nitrophenyl)ethan-1-ol (7). To a solution of oxirane **6** (400 mg, 1.63 mmol) in freshly distilled dry DMF (5 mL) was added NaN_3 (106 mg, 1.63 mmol), and the RM was stirred at 40 °C for 4 h. The RM was diluted with EtOAc, washed with water and brine solution, dried over Na_2SO_4, and evaporated. Flash column chromatography (hexane/EtOAc = 10:3) gave a yellow color oil **7** (120 mg, 40%). IR ν 3500, 3100, 2946, 2870, 2130, 1604, 1570, 1533, 1362, 1321, 1176, 1100, 1061, 907, 848, 758 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.11 (d, J = 2.2 Hz, 1H), 7.94 (d, J = 8.8 Hz, 1H), 7.64 (dd, J = 8.8 and 2.2 Hz, 1H), 5.55 (m, 1H), 3.77 (dd, J = 12.5 and 2.8 Hz, 1H), 3.45 (dd, J = 12.5 and 7.5 Hz, 1H), 2.74 (d, J = 3.9 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 146.02, 138.18, 132.22, 131.97, 129.40, 126.37, 68.80, 57.04. HRMS-ESI calcd for $C_8H_7BrN_4O_3Na$ [M + Na]$^+$ 284.96288, found 284.96597.

2-(2-Azido-1-(2-(2-bromoethoxy)ethoxy)ethyl)-4-bromo-1-nitrobenzene (8). To a stirred suspension of **7** (600 mg, 2.09 mmol) and cesium carbonate (3.39 g, 10.45 mmol) in freshly distilled dry acetonitrile (10 mL) was added bis (2-bromomethyl)ether (2.42 g, 10.45 mmol). The mixture was stirred for 5 h at 50 °C. Then, the RM was filtered, and the filtrate crude was evaporated using purified flash column chromatography (hexane/EtOAc = 10:2), which gave a yellow color oil **8** (400 mg, 53%). IR ν 2930, 2880, 2114, 1602, 1561, 1530, 1465, 1362, 1303, 1120, 957, 848, 757, 670 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.06 (d, J = 2.2 Hz, 1H), 7.93 (d, J = 8.6 Hz, 1H), 7.63 (dd, J = 8.6 and 2.2 Hz, 1H), 5.26 (dd, J = 7.2 and 2.8 Hz, 1H), 3.8−3.4 (m, 10H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 146.90, 137.05, 132.29, 132.19, 129.43, 126.48, 77.49, 71.27, 70.47, 69.52, 55.61, 30.20. HRMS-ESI calcd for $C_{12}H_{14}{}^{79}Br_2N_4O_4Na$ [M + Na]$^+$ 458.92740, found 458.92767.

2-(2-Azido-1-(2-(2-azidoethoxy)ethoxy)ethyl)-4-bromo-1-nitrobenzene (S2). A solution of **8** (400 mg, 0.91 mmol) and sodium azide (118 mg, 1.82 mmol) in dry DMF (4 mL) was stirred at RT for 16 h. Then the RM was diluted with EtOAc, washed with water and brine solution, dried over Na_2SO_4, and evaporated. Flash column chromatography (hexane/EtOAc = 10:3) gave a yellow color oil **S2** (295 mg, 80%). IR ν 2937, 2882, 2127, 1597, 1560, 1538, 1449, 1370, 1320, 1170, 848, 755 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.08 (d, J = 2.2 Hz, 1H), 7.95 (d, J = 8.7 Hz, 1H), 7.65 (dd, J = 8.7 and 2.2 Hz, 1H), 5.28 (dd, J = 7.2 and 2.9 Hz, 1H), 3.76−3.58 (m, 6H), 3.56−3.39 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 146.90, 137.04, 132.28, 132.17, 129.41, 126.48, 77.49, 70.58, 70.17, 69.55, 55.59, 50.68 cm^{-1}. HRMS-ESI calcd for $C_{12}H_{14}{}^{79}BrN_7O_4Na$ [M + Na]$^+$ 422.01829, found 422.01773.

2-(2-(2-Aminoethoxy)ethoxy)-2-(5-bromo-2-nitrophenyl)ethan-1-amine (9). To a solution of compound **S2** (520 mg, 1.3 mmol) in THF (5 mL) was added PPh_3 (1.02 g, 3.9 mmol), and the RM was stirred for 12 h at RT. Then H_2O (0.5 mL) was added, and the resulting solution was stirred for 12 h. The solvent was evaporated, and the reaction products were purified using flash column chromatography (CH_2Cl_2/MeOH/Et_3N = 7:2:1), which gave a yellow color liquid **9** (360 mg, 80%). IR ν 3442, 2933, 1531, 1359, 1129, 845, 658 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.99 (d, J = 2.1 Hz, 1H), 7.92 (d, J = 8.7 Hz, 1H), 7.61 (dd, J = 8.7 and 2.1 Hz, 1H), 5.1 (dd, J = 8.1 and 2.6 Hz, 1H), 3.72−3.51 (m, 6H), 3.33−2.82 (m, 4H), 2.03 (bs, 2H), 1.41 (t, J = 7.5 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 147.22, 138.14, 131.95, 131.68, 129.20, 126.45, 77.23, 71.1, 70.12, 69.13, 47.33, 40.88. HRMS-ESI calcd for $C_{12}H_{18}{}^{79}BrN_3O_4Na$ [M + Na]$^+$ 370.03729, found 370.03726.

Diethyl-5-(5-bromo-2-nitrophenyl)-3,12-bis(2-ethoxy-2-oxoethyl)-6,9-dioxa-3,12-diazatetradecanedioate (1a). To a solution of **9** (800 mg, 2.3 mmol), sodium iodide (3.45 g, 23.05 mmol), and diisopropyl ethylamine (1.48 g, 11.52 mmol) in dry CH_3CN (30 mL), ethyl bromoacteate (3.85 g, 23.05 mmol) was added dropwise at 0 °C, and the RM was stirred for

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3 h at 80 °C. The RM was filtered, and the filtrate was evaporated. Flash column chromatography (hexane/EtOAc = 2:1) gave a pale yellow color oil **1a** (1.1 g, 70%). IR ν 2985, 1752, 1600, 1530, 1211, 1045, 759, 602 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.98 (d, J = 2.1 Hz, 1H), 7.86 (d, J = 8.7 Hz, 1H), 7.54 (dd, J = 8.7 and 2.1 Hz, 1H), 5.2 (dd, J = 7.2 and 3.3 Hz, 1H), 4.19−4.11 (m, 8H), 3.78−3.67 (m, 4H), 3.62−3.38 (m, 10H), 3.09−2.91 (m, 4H) 1.29−1.22 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.70, 171.41, 146.97, 139.13, 132.10, 131.43, 128.98, 126.16, 77.80, 70.32, 70.18, 69.14, 61.17, 60.45, 55.98, 55.73, 53.84, 14.27. HRMS-ESI calcd for $C_{28}H_{42}BrN_3O_{12}Na$ $[M + Na]^+$ 714.18441, found 714.18518.

Diethyl-5-(5-benzofuran-2-yl)-2-nitrophenyl)-3,12-bis(2-ethoxy-2-oxoethyl)-6,9-dioxa-3,12-diazatetradecanedioate (2a). A solution of **1a** (200 mg, 0.28 mmol), benzofuran-2-boronic acid (52.4 mg, 0.31 mmol), $Pd(PPh_3)_4$ (16.7 mg, 0.14 mmol), and potassium carbonate (52 mg, 0.31 mmol) was taken in 10 mL THF/H_2O (9:1) in a round-bottom flask and was stirred for 3 h at 60 °C. Then, the reaction mixture was filtered. The filtrate was washed with water, extracted with EtOAc organic layer, dried over Na_2SO_4, evaporated under reduced pressure, and purified using column chromatography to afford the desire compound as a yellow oil **2a** (95%). IR ν 2974, 2903, 2859, 1730, 1610, 1515, 1445, 1346, 1255, 1178, 1024, 746. ^1H NMR (400 MHz, $CDCl_3$): δ 8.29 (s, 1H), 8.1 (d, J = 8.6 Hz, 1H), 7.9 (dd, J = 2.0 and 8.6 Hz, 1H), 7.68 (d, J = 7.7 Hz, 1H), 7.6 (d, J = 8.1 Hz, 1H), 7.4−7.29 (m, 3H), 5.34−5.3 (m, 1H), 4.22−4.07 (m, 8H), 3.8 (s, 4H), 3.65−3.5 (m, 10H), 3.17−2.9 (m, 4H), 1.31−1.3 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.82, 171.34, 170.19, 155.42, 147.24, 128.74, 125.68, 124.77, 124.04, 123.45, 121.65, 111.53, 105.18, 78.16, 77.35, 77.24, 77.03, 76.72, 70.29, 69.06, 61.30, 60.42, 55.87, 55.75, 53.71, 21.08, 14.28, 14.22. HRMS-ESI calcd for $C_{36}H_{47}N_3O_{13}Na$ $[M + Na]^+$ 752.30011, found 752.30011.

Diethyl-5-(4′-(dimethylamino)-4-nitro-[1,1′-biphenyl]-3-yl)-3,12-bis(2-ethoxy-2-oxoethyl)-6,9-dioxa-3,12-diazatetradecanedioate (3a). A solution of **1a** (200 mg, 0.28 mmol), 4-(*N*,*N*-dimethyl amino)phenylboronic acid (52.4 mg, 0.31 mmol), $Pd(PPh_3)_4$ (16.7 mg, 0.14 mmol), and potassium carbonate (60 mg, 0.43 mmol) was taken in 10 mL THF/H_2O (9:1) in a round-bottom flask and was stirred for 3 h at 60 °C. Then, the reaction mixture was filtered. The filtrate was washed with water, extracted with EtOAc organic layer, dried over Na_2SO_4, evaporated under reduced pressure, and purified using column chromatography to afford the desire compound as a yellow oil **3a** (90%): ^1H NMR (400 MHz, $CDCl_3$): δ 8.27 (d, J = 2.1 Hz, 1H), 7.84 (d, J = 8.6 Hz, 1H), 7.64−7.6 (m, 2H), 7.19 (dd, J = 2.2 and 2.1 Hz, 1H), 6.71−6.67 (m, 2H), 5.32 (dd, J = 8.3 and 2.3 Hz, 1H), 4.03−3.84 (m, 12H), 3.65−3.62 (m, 4H), 3.48−3.2 (m, 8H), 3.16−2.85 (m, 8H), 0.95 (t, J = 7.2 Hz, 6H), 0.89 (t, J = 7.2 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.80, 171.31, 150.95, 146.67, 145.60, 137.69, 128.11, 125.83, 125.68, 125.25, 124.89, 112.49, 78.28, 77.22, 70.33, 70.24, 68.83, 61.46, 60.39, 60.37, 55.88, 55.68, 53.73, 40.31, 14.28, 14.22. HRMS-ESI calcd for $C_{36}H_{52}N_4O_{12}Na$ $[M + Na]^+$ 755.34739, found 755.34631.

Bis(acetoxymethyl) 3,12-Bis(2-(acetoxymethoxy)-2-oxoethyl)-5-(5-(benzofuran-2-yl)-2-nitrophenyl)-6,9-dioxa-3,12-diazatetradecanedioate (2b). To a compound **2a** (50 mg, 0.068 mmol) in 3 mL of MeOH was added potassium hydroxide (KOH) (19.2 mg, 0.34 mmol) at 0 °C. The RM was stirred for 2 h at 60 °C, evaporated under reduced pressure, and dried over. The yellow color solid obtained was dissolved in freshly distilled dry DMF (3 mL). Diisopropylethyl amine (52 mg, 0.4 mmol) and bromomethylacetate (166 mg, 1.08 mmol) were added at 0 °C. After 16 h of stirring at RT, the RM was diluted with water and extracted with ethyl acetate. The organic layer was washed with water and brine solution, dried over Na_2SO_4, and evaporated under reduced pressure. The reaction products were purified using silica gel column chromatography and further purified by recycling HPLC with gel permeation chromatography (GPC) column to give pure AM ester as a yellow color oil **2b** (60%). ^1H NMR (400 MHz, $CDCl_3$): δ 8.27 (d, J = 2.0 Hz, 1H), 8.16 (d, J = 8.7 Hz, 1H), 7.93 (dd, J = 8.6 and 2.0 Hz, 1H), 7.68 (d, J = 8.6 Hz, 1H), 7.61 (d, J = 8.2 Hz, 1H), 7.4−7.37 (m, 1H), 7.31−7.24 (m, 2H), 5.79 (s, 4H), 5.9 (s, 4H), 5.31 (dd, J = 7.9 and 2.45 Hz, 1H), 3.94−3.84 (m, 4H), 3.67−3.47 (m, 10H), 3.23−3.09 (m, 2H), 2.99−2.86 (m, 2H), 2.13 (s, 6H), 2.08 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.51, 170.01, 169.73, 169.51, 155.45, 153.30, 147.12, 137.80, 135.66, 132.12, 132.10, 128.61, 128.58, 125.74, 124.52, 124.11, 123.53, 121.60, 111.57, 105.30, 79.14, 78.17, 77.25, 70.34, 70.29, 69.05, 61.10, 55.43, 55.36, 53.53, 20.77, 20.71. HRMS-ESI calcd for $C_{40}H_{47}N_3O_{21}Na$ $[M + Na]^+$ 928.25943, found 928.25897.

Bis(acetoxymethyl) 3,12-Bis(2-(acetoxymethoxy)-2-oxoethyl)-5-(4′-(dimethylamino)-4-nitro-[1,1′-biphenyl]-3-yl)-6,9-dioxa-3,12-diazatetradecanedioate (3b). To the compound **3a** (50 mg, 0.068 mmol) in 3 mL of MeOH was added KOH (19.1 mg, 0.34 mmol) at 0 °C. The RM was stirred for 2 h at 60 °C, evaporated under reduced pressure, and dried over. The yellow color solid obtained was dissolved in freshly distilled dry DMF (3 mL). Diisopropylethyl amine and bromomethylacetate were added at 0 °C. After 16 h of stirring at RT, the RM was diluted with water and extracted with ethyl acetate. The organic layer was washed with water and brine solution, dried over Na_2SO_4, and evaporated under reduced pressure. The reaction products were purified using silica gel column chromatography and further purified by recycling HPLC with GPC column to give pure AM ester as a yellow color oil **3b** (60%). ^1H NMR (400 MHz, $CDCl_3$): δ 8.09 (d, J = 8.7 Hz, 1H), 7.96 (d, J = 2.1 Hz, 1H), 7.63−7.57 (m, 3H), 6.84−6.8 (m, J = 8.9, 2H), 5.79 (s, 4H), 5.72 (s, 4H), 5.32 (dd, J = 8.3 and 1.8 Hz, 1H), 3.91 (s, 4H), 3.65−3.44 (m, 10H), 3.23−3.02 (m, 8H), 2.97−2.84 (m, 2H), 2.14 (s, 6H), 2.1 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.63, 170.10, 169.71, 169.58, 150.97, 146.71, 145.45, 137.41, 128.19, 125.84, 125.63, 125.03, 112.46, 79.12, 78.25, 77.23, 70.31, 68.74, 61.19, 55.41, 55.20, 53.63, 40.31, 20.79, 20.72. HRMS-ESI calcd for $C_{40}H_{52}N_4O_{20}Na$ $[M + Na]^+$ 931.30671, found 931.30420.

5-(5-(Benzofuran-2-yl)-2-nitrophenyl)-3,12-bis-(carboxymethyl)-6,9-dioxa-3,12-diazatetradecanedioic acid (2c). Tetraethyl ester **2a** (100 mg, 0.13 mmol) was dissolved in methanol (4 mL) and then saponified by the addition of KOH (38.4 mg, 0.68 mmol) and stirring for 2 h at 60 °C. After evaporation of methanol and acidification with 2 N aqueous HCl to pH 2, the yellow solid obtained was collected and dried to afford **2c** (92%). ^1H NMR (400 MHz, CD_3OD): δ 8.32−8.30 (bs, 1H), 8.24 (d, J = 8.6 Hz, 1H), 8.13 (dd, J = 1.8 and 8.6 Hz, 1H), 7.71 (d, J = 7.5 Hz, 1H), 7.62−7.56 (m, 2H), 7.4−7.28 (m, 2H), 5.65 (m, 1H), 4.5 (bs, 4H), 4.15 (bs, 4H), 4.0−3.5 (m, 10H). ^{13}C NMR (100 MHz, CD_3OD): 168.86, 168.47, 157.06, 154.37, 148.86, 137.65, 135.27, 130.31, 127.77, 127.47, 127.27, 125.71, 125.05, 123.30, 112.58, 107.5, 74.69, 71.48, 69.75, 66.85, 66.81, 60.87, 57.43, 57.38, 56.66, 54.02.

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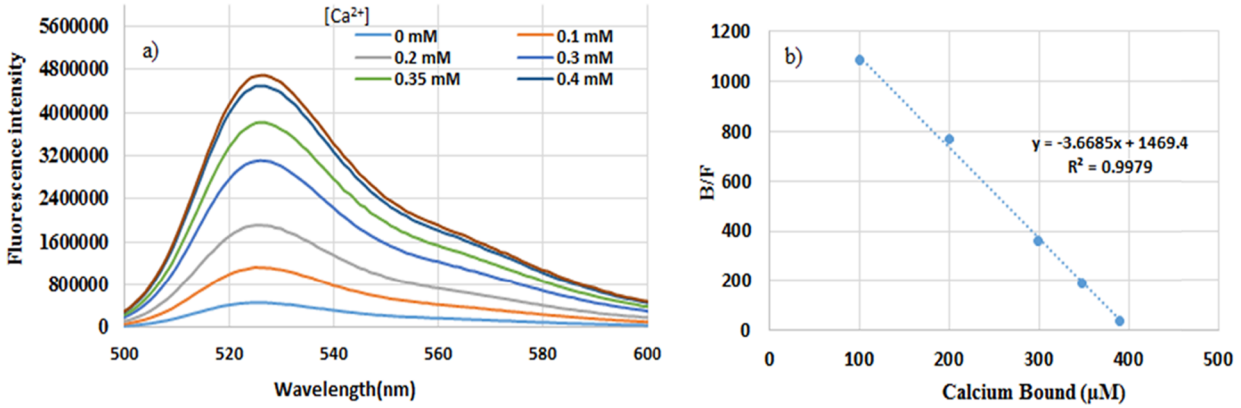


Figure 5. (a) Emission spectra of fluo-3 (10 μM, excitation at 490 nm) resulting from the titration of **2c** (1.0 mM in Hepes/100 mM KCl, pH 7.4 at 298 K) with incremental addition of Ca^{2+}. (b) Scatchard analysis (using K_d value of 500 nM for fluo-3) of these data.

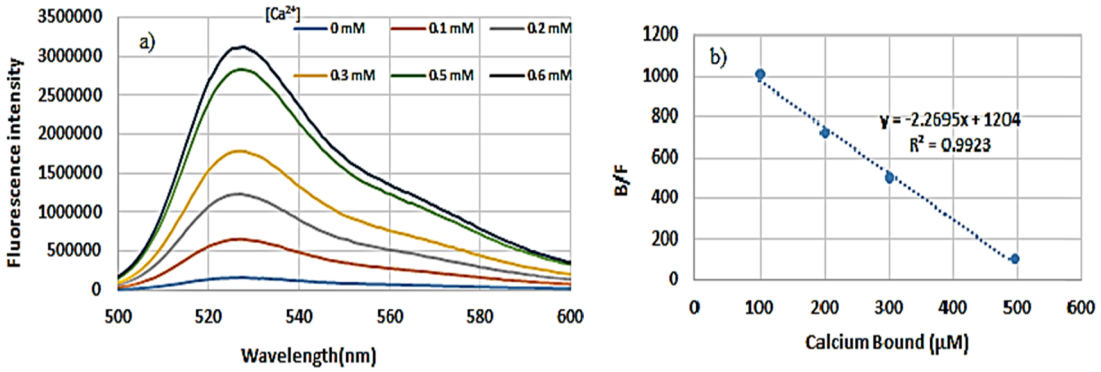


Figure 6. (a) Emission spectra of fluo-3 (10 μM, excitation at 490 nm) resulting from the titration of **3c** (1.0 mM in Hepes/100 mM KCl, pH 7.4 at 298 K) with incremental addition of Ca^{2+}. (b) Scatchard analysis (using K_d value of 500 nM for fluo-3) of these data.

HRMS-ESI calcd for $C_{28}H_{31}N_3O_{13}$ $[M - H]^+$ 616.17841, found 616.17767.

3,12-Bis(carboxymethyl)-5-(4′-(dimethylamino)-4-nitro-[1,1′-biphenyl]-3-yl)-6,9-dioxa-3,12-diazatetradecanedioic acid (3c). Tetraethyl ester **3a** (100 mg, 0.13 mmol) was dissolved in methanol (4 mL) and then saponified by the addition of KOH (38.3 mg, 0.68 mmol) and stirring for 2 h at 60 °C. After evaporation of methanol and acidification with 2 N aqueous HCl to pH 2, the yellow solid obtained was collected and dried to afford **3c** (90%). ^{1}H NMR (400 MHz, CD_3OD): δ 8.15 (d, J = 8.4 Hz, 1H), 8.05 (s, 1H), 7.95−7.85 (m, 3H), 7.81−7.65 (m, 2H), 5.62−5.58 (m, 1H), 4.46 (s, 2H), 4.45 (s, 2H), 4.17 (s, 2H), 4.16 (s, 2H), 3.94−3.73 (m, 4H), 3.66−3.45 (m, 6H), 3.27 (s, 6H). ^{13}C NMR (100 MHz, CD_3OD): 168.72, 168.42, 149.21, 146.64, 145.04, 141.1, 134.77, 130.94, 130.05, 128.67, 127.68, 112.80, 74.67, 71.44, 69.73, 66.91, 60.91, 57.34, 56.67, 47.27. HRMS-ESI calcd for $C_{28}H_{36}N_4O_{12}$ $[M + H]^+$ 621.24025, found 621.24084.

Calcium Binding Affinities. The association constant for the two chelators **2c** and **3c** was calculated using the method of Ellis-Davies et al. by the equation

$$[Ca^{2+}]_B/[Ca^{2+}]_F = K_a([\text{chelator}]_T - [Ca^{2+}]_B)$$

Here $[Ca^{2+}]_B$ is the concentration of Ca^{2+} bound to the chelator, $[Ca^{2+}]_F$ is the concentration of the free Ca^{2+}, and $[\text{chelator}]_T$ is the total concentration of the chelator (NPBF-EGTA acid or BP-EGTA acid) in the solution. The free and bound calcium concentrations were obtained from the emission spectra of a calcium-sensitive dye (fluo-3, K_d = 500 nm) through titration with calcium chloride solution.

In this measurement, chelators (1.0 mM), fluo-3 (0.01 mM), Hepes (10 mM) pH 7.4, and 100 mM KCl solutions were used. A 3.0 mL solution of 1.0 mM chelator was used. The solution was buffered to pH 7.4 with Hepes, and the ionic strength was set with 100 mM KCl. Data from the titration with incremental addition of Ca^{2+} are shown in Figures 5 and 6.

$[Ca^{2+}]_B/[Ca^{2+}]_F$ were obtained from the absorption curves shown in Figures 5 and 6. The concentration of chelators was 1.0 mM, and the $CaCl_2$ concentrations were 0.1, 0.2, 0.3, 0.35, 0.4, and 0.5 mM for NPBF-EGTA acid and 0.1, 0.2, 0.3, 0.5, and 0.6 mM for BP-EGTA acid. After these concentrations, any change was not observed in the absorption curve.

The free calcium concentrations were calculated from the equation

$$[Ca^{2+}]_F = K_d(A_{int} - A_{min})/(A_{max} - A_{int})$$

where K_d = 500 nM (fluo-3), A_{int} = absorbance of chelator at setted Ca^{2+} concentration, A_{min} = absorbance of chelator without Ca^{2+}, and A_{max} = absorbance of chelator with excess Ca^{2+}.

Dissociated Cultured Neurons, Electrophysiology, and Two-Photon Uncaging. Cerebral cortices from male C57BL/6 mouse brains (E17.5) with 0.15% trypsin in ethylenediaminetetraacetic acid (Invitrogen, Carlsbad, CA, USA) and DNase I (30 mg mL^{-1}) were used. The cells were plated on laminin- and poly-D-lysine-coated cover glasses with a density of 1.7 × 10^5 cells per square centimeter. The neurons

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were maintained in a serum-free medium (Neurobasal Medium; Invitrogen) supplemented with 1% GlutaMAX and 2% B-27 supplement (Invitrogen). For electrophysiological experiments, an extracellular solution was based on SolA containing 10 mM $CaCl_2$, 100 mM cyclothiazide, 50 mM picrotoxin, and 320 nM Na-pyruvate, at pH 7.4. The cultured cells were first loaded with the compound **2a** (100 μM) for 40 min in a solution containing CrEL (0.03%, Invitrogen) and bovine serum albumin (5%) in SolA, followed by washing with SolA for 20 min at 35 °C. The postsynaptic cells (DIV 10−15) were whole-cell-clamped (holding potential = −70 mV) and perfused with intracellular solution (in mM: CsCl 140, Hepes 10, MgATP 4, Na_2GTP 0.3, Na_2-phosphocreatine 10, Alexa-594 0.025, pH = 7.25 with CsOH). Electrical stimulation of 70 μA amplitude was applied, at 100−150 μm from the dendrites.

Two-Photon Excitation Imaging and Uncaging. The cultured neurons were placed on an inverted laser-scanning microscope (FV1000 and IX81; Olympus Corporation, Tokyo, Japan) equipped with a water-immersion objective lens (UPlanApo60xW/IR; N.A. 1.2; Olympus Corporation) and a femtosecond laser (Mai Tai; Spectra Physics Inc., Mountain View, CA, USA). Tunable wavelengths were set at 950 and 720 nm for imaging and uncaging, respectively.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.6b00119.

Experimental procedures and compound characterization (PDF)

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Notes

The authors declare no competing financial interest.

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