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ORIGINAL PAPER

Synthesis, Spectroscopic Properties, and Biological Applications of Eight Novel Chlorinated Fluorescent Proteins-labeling Probes

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Abstract Eight novel chlorinated fluorescent proteins-labeling probes with a linker and reactive group were prepared in 7 steps by the reaction of chlorinated resorcinols with 3, 6-dichloro-4-carboxyphthalic anhydride in the presence of methanesulfonic acid. Structures of target compounds and intermediates were determined *via* IR, MS, ^{1}H NMR and element analysis. The spectral properties of the chlorinated fluoresceins were studied. These fluorescent probes showed absorbance peaks at 508–536 nm and fluorescence peaks at 524–550 nm. It was found that they have absorption and emission maxima at long wavelengths and high fluorescence quantum yields. Emission spectra of chlorinated fluoresceins shifted towards long wavelength with increase in chlorine. The probes were used for fluorescence imaging of cells in order to investigate whether they can conjugate to cells. The fluorescence imaging of living cells showed that they were localized in cell nucleus. However, they were localized in cytosol of chemically fixed cells. These probes will be useful reagents for the preparation of stable fluorescent conjugates.

Keywords Fluorescent dye · Probe dye · Cell labeled · Spectroscopic properties · Synthesis

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Introduction

For applications in imaging and microscopy, the most important characteristics of a fluorescent dye and its bio-conjugates are: the position, form, fluorescence quantum yield, and lifetime of the excited state of a free dye and its bio-conjugates [1]. Carboxy-functionalized fluorescein and rhodamine dyes are useful reagents for the preparation of hydrolytically stable fluorescent conjugates [2]. They are powerful tools for exploring cellular biology owing to their brightness, high quantum yields, low-energy excitation and emission wavelengths, and biocompatibility [3].

A wide variety of fluorescein derivatives have been prepared and used as fluorescent detection reagents. Fluorescently labeled biologically active molecules have many important analytical and biochemical applications. Many fluorescein derivatives have functional groups that are suitable for reaction with other molecules, and can therefore serve as labels in a variety of analytical applications ranging from probing cell functions to monitoring the level of drugs in human fluids via immunoassays [4]. Fluorescein derivatives are particularly suitable for, but not limited to, biological experiments in which fluorescein is covalently attached to materies such as peptides, proteins, nucleotides, oligonucleotides, drugs, hormones, lipids, and other biomolecules [5–10].

Although these dyes have a same xanthene-based skeleton, different substituents can be made to cause marked differences in absorbance and fluorescence emission wavelengths. Selective substitution of chlorine for aromatic hydrogen has been seen to increase fluorescence efficiency and to narrow absorbance and emission maxima compared with fluorescein. These characteristics are very useful for multi-color imaging [11].

The selective substitution of chlorine for aromatic hydrogen in organic compounds results in profound changed in their photophysical properties. Therefore, it is very important to study the fluorescein derivatives substituted by chlorine [12].

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Several chlorinated fluoresceins such as 4, 5, 6, 7-tetrachlorofluorescein, 2′, 7′-dichloro-4, 5, 6, 7-tetrachlorofluorescein, 4′, 5′-dichloro-4, 5, 6, 7-tetrachlorofluorescein, and 2′, 4′, 5′, 7′-tetrachloro-4, 5, 6, 7-tetrachlorofluorescein have been synthesized by our group. They were found to have absorption and emission maxima at long wavelengths and high fluorescence quantum yields [13].

In an effort to facilitate and improve conjugations, we have added a spacer linker between the chlorinated fluorophore and the reactive group. 4-Aminobutanoic acid or 3-amiopropanoic acid can be used as a spacer linker. This allows us to prepare succinimidyl ester derivatives, the most amine-selective and stable reactive group for labeling proteins.

However, to our knowledge, the preparation and spectroscopic properties of chlorinated fluorescein have not been reported detailedly and systematically. Four probes were reported briefly in a form of letter by our group [14, 15].

The present work details, for the first time, the preparation of eight novel chlorinated fluorescent proteins-labeling probes. Spectroscopic properties and biological applications of these novel chlorinated fluorescent proteins-labeling probes are also described in this work.

Experimental Procedures

Materials Unless mentioned otherwise, all chemicals, reagents and solvents for the synthesis of the compounds were analytically pure grade and purchased from commercial sources. All organic solvents were anhydrous grade. 3, 6-dichloro-4-carboxy-phthalic anhydride was prepared by our group.

Instrumentation All fluorescence measurements were carried out at room temperature on a Hitachi F-4500 spectrofluorimeter equipped with a xenon lamp source and a 10 mm quartz cell. Absorption spectra were recorded on a Hitachi U-3310 UV-vis spectrophotometer using a 10 mm quartz cell. ^{1}H NMR spectra were recorded on a Varian Inova-400 spectrometer operating at 400 MHz using either $CDCl_3$ or DMSO-d_6 as solvents. MALDI-TOF mass spectra were recorded using an AXIMA-CFR™ plus mass spectrometer. For MALDI-TOF analysis, samples were prepared by dissolving in methanol and spotting 1 μL of the solution. Infrared spectra were obtained as KBr pellets on an EQUINOX-55 FTIR spectrometer.

Cell Culture MC3T3-E1 murine calvarial preosteoblasts were obtained from the American Type Culture Collection. The MC3T3-E1 cells were cultured in Dulbecco's modified eagle's medium (Gibco, Grand Island, NY, USA), supplemented with 10 % fetal calf serum, 1 % penicillin, and 1 % streptomycin at 37 °C in an atmosphere of 95 % oxygen and 5 % CO_2 [16–18].

Cells were plated at a cell density of 1×10^4 cells/well on glass coverslips placed at the bottom of 24-well plates and incubated at 37 °C (5 % CO_2) overnight.

Preparation of Living Cell for Fluorescent Imaging

After incubation overnight to allow the cells to attach on the plate, the culture media was discarded, and 0.1 mL of the probe solution (1×10^{-5} mol/L in DMEM) was added to each well, followed by incubation at 37 °C for 45 min. The supernatant was abandoned. Then, the cells were washed gently twice with sterilized PBS to remove excess dye before plate sealing and imaging.

Preparation of Fixed Cell for Fluorescent Imaging

After incubation overnight to allow the cells to attach on the plate, the culture media was discarded, and incubate the cells with 4 % PFA in PBS for 10 min at rt. Rinsed the fixed cells three times in PBS, and 0.1 mL of the probe solution (1×10^{-5} mol/L in PBS) was added to each well. Then, stained the cells at rt for 30 min. Rinsed cells gently with PBS several times to remove excess dye before plate sealing and imaging.

Images were taken using a Nikon Eclipse 80i microscope equipped for epifluorescence using the following filter sets: DAPI(Blue: DAPI channel); FITC (Oregon Green channel); For experiments that evaluated the intracellular distribution of fluorescent probes, the microscope parameters, including fluorescence intensity and exposure, were optimized for each fluorophore.

Synthesis

Synthesis of 5(6)-carboxy-4, 7-dichlorofluorescein **(1a)**

The mixture of 3, 6-dichloro-4-carboxyphthalic anhydride (2.61 g, 10 mmol) and resorcinol (2.53 g, 23 mmol) in methanesulfonic acid (50 mL) was heated and stirred under nitrogen at 50 °C for 4 h then at 110 °C for 24 h. After cooling to room temperature, the mixture was poured into the ice-water. The precipitation was filtered and washed several times with 0.1 M HCl. The solid was dried in air overnight or in vacuum to constant weight. The mixture was separated by column chromatography on silica with the eluent of 1:10:80 glacial acetic acid/methanol/chloroform. The compound **1a** (3.74 g) was obtained as a green black powder. $C_{21}H_{10}Cl_2O_7$, yield: 84 %, ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 10.15(s, 1H, -

COOH), 6.85(d, 2H 1′ and 8′-ArH), 6.66(s, 2H, 4′and 5′-ArH), 6.55(d, 2H, 2′ and 7′-ArH). MALDI-TOF MS, m/z: 445.39(Calcd: 445.21). FT-IR(KBr), υ/cm^{-1}: 3476, 1765, 1609, 1487, 1433.

Synthesis of 2′, 7′-dichloro-5(6)-carboxy-4, 7-dichlorofluorescein **(1b)**

This compound was prepared from 3, 6-dichloro-4-carboxyphthalic anhydride (2.61 g, 10 mmol) and 4-chlororesorcinol (3.32 g, 23 mmol) by the procedure utilized for compound **1a**. Compound **(1b)** (4.01 g) was obtained as a tan powder. $C_{21}H_8Cl_4O_7$, yield: 78 %, ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.12(s, 1H, -COOH), 7.21(s, 2H, 4′ and 5′ -ArH), 6.87(s, 2H, 1′ and 8′ -ArH). MALDI-TOF MS, m/z: 515.55(Calcd: 514.10). FT-IR(KBr), υ/cm^{-1}: 3421, 1759, 1608, 1434, 1175.

Synthesis of 4′, 5′-dichloro-5(6)-carboxy-4, 7-dichlorofluorescein **(1c)**

This compound was prepared from 3, 6-dichloro-4-carboxyphthalic anhydride (2.61 g, 10 mmol) and 2-chlororesorcinol (3.32 g, 23 mmol) by the procedure utilized for compound **1a**. Compound **(1c)** (4.06 g) was obtained as an orange powder. $C_{21}H_8Cl_4O_7$, yield: 79 %, ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.05(s, 1H, -COOH), 6.93(d, 2H, 1′ and 8′-ArH), 6.82(d, 2H, 2′ and 7′-ArH). MALDI-TOF MS, m/z: 514.76 (Calcd: 514.10). FT-IR(KBr), υ/cm^{-1}: 3432, 1760, 1605, 1433, 1213.

Synthesis of 2′, 4′, 5′, 7′-tetrachloro-5(6)-carboxy-4, 7-dichlorofluorescein **(1d)**

This compound was prepared from 3, 6-dichloro-4-carboxyphthalic anhydride (2.61 g, 10 mmol) and 2, 4-dichlororesorcinol (4.12 g, 23 mmol) by the procedure utilized for compound **1a**. Compound **(1d)** (4.26 g) was obtained as a brick red powder. $C_{21}H_6Cl_6O_7$, yield: 73 %, ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.15(s, 1H, -COOH), 8.54(s, 1H, ArOH), 8.22(s, 1H, 5-ArH), 6.86 (s, 2H, 1′ and 8′-ArH). MALDI-TOF MS, m/z: 581.90 (Calcd: 582.99). FT-IR(KBr), υ/cm^{-1}: 3480, 1768, 1709, 1478, 1433, 1212, 997.

Synthesis of 6-carboxy-4, 7-dichlorofluorescein dipivalate diisopropylamine salt **(2a)**

5(6)-carboxy-4, 7-dichlorofluorescein (**1a**) (6.68 g, 15 mmol) was refluxed in trimethylacetic anhydride (28 mL) for 2 h. The reaction mixture was cooled to room temperature and diluted with tetrahydrofuran (THF 40 mL) and water (40 mL). After 2 h of vigorous stirring, ether (50 mL) was added and the aqueous layer was removed. The organic layer was washed with phosphate buffer (3×50 mL, 1.4 M, pH 7.0), aqueous HCl (50 mL, 1 M), and saturated NaCl and dried with $MgSO_4$. The solvent was removed by evaporation, and resulting yellow syrup was dissolved in anhydrous ethanol (50 mL). Diisopropylamine (5.0 mL, 36 mmol) was added, and the mixture was cooed to −20 °C in freezer overnight. The resulting solid was removed by filtration and washed with cold ethanol and acetone to give white powder 4.23 g (39.5 %) of compound **2a**, which had a isomeric purity >95 % as determined by HPLC analysis. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.84(d, 2H, 1′ and 8′-ArH), 6.81(d, 2H, 2′ and 7′-ArH), 7.71(s, 1H, 5-ArH), 3.40(q, 2H, -CH=), 1.36(s, 18H, -tBuH), 1.25(s, 12H, *i*-propyl H). MALDI-TOF MS, m/z: 714.41 (Calcd: 714.63). FT-IR(KBr), υ/cm^{-1}: 3423, 2873, 2723, 1777, 1610, 1583, 1497, 1392, 1225, 1119, 894.

Synthesis of 2′, 7′-dichloro-6-carboxy-4, 7-dichlorofluorescein dipivalate diisopropylamine salt **(2b)**

This compound was prepared from compound **1b** according to the procedure described above for compounds **2a**. The compound **2b** (3.68, 36 %) was obtained as a white powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.40(s, 1H) 7.52 (s, 2H), 7.49(s, 2H), 3.44(a quintet, 2H), 1.34(s, 18H), 1.19(d, 12H). MALDI-TOF MS, m/z: 783.10 (calcd: 783.52). FT-IR(KBr), υ/cm^{-1}: 3440, 2979, 1767, 1640, 1094; Anal. Calcd. for $C_{39}H_{39}NO_2Cl_4$: C 56.71, H 5.02, N 1.79; found: C 56.73, H 5.05, N 1.74.

Synthesis of 4′, 5′-dichloro-6-carboxy-4, 7-dichlorofluorescein dipivalate diisopropylamine salt **(2c)**

This compound was prepared from compound **1c** according to the procedure described above for compounds **2a**. The compound **2c** (4.09, 40 %) was obtained as a white powder. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.40 (s, 1H), 7.38(d, 2H), 7.26(d, 2H), 3.44(a quintet, 2H), 1.36 (s, 18H), 1.19(d, 12H). MALDI-TOF MS, m/z: 783.41 (calcd: 783.52). FT-IR(KBr), υ/cm^{-1}: 3424, 2977, 1781, 1640, 1424, 1092. Anal. Calcd. for $C_{39}H_{39}NO_2Cl_4$: C 56.71, H 5.02, N 1.79; found: C 56.93, H 5.08, N 1.83.

Synthesis of 2′, 4′, 5′, 7′-tetrachloro-6-carboxy-4, 7-dichlorofluorescein dipivalate diisopropylamine salt **(2d)**

This compound was prepared from compound **1d** according to the procedure described above for compounds **2a**. The compound **2d** (4.94, 38.6 %) was obtained as a white powder. ^{1}H

NMR ($CDCl_3$, 400 MHz) δ: 6.87 (s, 2H, 1′ and 8′-ArH), 7.81 (s, 1H, 5-ArH), 3.25 (s, 2H, $–NH_2$), 1.44 (s, 18H, t-BuH), 1.24 (s, 12H, i-propyl H). MALDI-TOF MS, m/z: 852.52, (calcd: 852.41). FT-IR(KBr), υ/cm^{-1}: 2979, 2874, 1775, 1642, 1425, 1214, 1075, 682.

Synthesis of 6-carboxy-4, 7-dichlorofluorescein dipivalate **(3a)**

Carboxyfluorescein salt **2a** (2.89 g, 4 mmol) was dissolved in CH_2Cl_2 (50 mL) and extracted with aqueous HCl (1 M, 3×50 mL). The organic layer was dried with Na_2SO_4, and the solvent was evaporated to give 2.21 g of compound **3a**. Yield 90 %, white powder, ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.87(s, 2H, 1′ and 8′-ArH), 6.84(d, 2H, 2′ and 7′-ArH), 8.11(s, 1H, 5-ArH), 1.37(s, 18H, -tBuH). MALDI-TOF MS, m/z: 613.30, (Calcd: 613.44). FT-IR(KBr), υ/cm^{-1}: 3445, 2975, 1784, 1612, 1496, 1423, 1398, 1157,1116, 1027, 877.

Synthesis of 2′, 7′-dichloro-6-carboxy-4, 7-dichlorofluorescein dipivalate **(3b)**

This compound was prepared from compound **2b** according to the procedure described above for compound **3a**. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.18(s, 1H), 7.13(s, 2H), 6.91 (s, 2H),1.40(s, 18H). MALDI-TOF MS, m/z: 682.89 (calcd: 682.33). FT-IR(KBr), υ/cm^{-1}: 2976, 1765, 1478, 1408, 1093. Anal. Calcd. for $C_{31}H_{24}O_9Cl_4$: C 52.59, H 3.10; Found: C 52.73, H 3.05.

Synthesis of 4′, 5′-dichloro-6-carboxy-4, 7-dichlorofluorescein dipivalate **(3c)**

This compound was prepared from compound **2c** according to the procedure described above for compound **3a**. Yield, 89.5 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.13(s, 1H) 6.94(d, 2H), 6.81 (d, 2H), 1.42(s, 18H). MALDI-TOF MS, m/z: 682.45(calcd: 682.33). FT-IR(KBr), υ/cm^{-1}: 2976, 1765, 1478, 1425, 1092. Anal. Calcd. for $C_{31}H_{24}O_9Cl_4$: C 52.59, H 3.10; Found: C 52.63, H 3.25.

Synthesis of 2′, 4′, 5′, 7′-tetrachloro-6-carboxy-4, 7-dichlorofluorescein dipivalate **(3d)**

This compound was prepared from compound **2d** according to the procedure described above for compound **3a**. Yield, 88 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.87(s, 2H, 1′ and 8′-ArH), 8.20(s, 1H, 5-ArH), 1.44(s, 18H, -tBuH). FT-IR(KBr), υ/cm^{-1}: 2977, 1778, 1589, 1454, 1425, 1369, 1212, 1078, 873. Anal. Calcd for $C_{31}H_{22}Cl_6O_9$: C 49.56, H 2.95; Found: C 49.52, H 2.94.

Synthesis of 4, 7-dichloro-dipivaloyl-6-carboxyfluorescein N-hydroxysuccinimidyl ester **(4a)**

Anhydrous pyridine (16 mL) and N-hydroxysuccinimidyl trifluoroacetate (NHS-TFA) (8.44 g, 40 mmol) were add to a solution of compound **3a** (3.07 g, 5 mmol) in anhydrous CH_2Cl_2 under vigorous stirring. The resultant mixture was stirred at room temperature for 5 h. TLC ($CHCl_3$/ MeOH = 10:1) was used to confirm the completion of the reaction. The mixture was washed with 4 % HCl (2×100 mL) and saturated saline (2×50 mL), dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure, and residue was dried in vacuum to yield compound **4a** (2.84 g, 80 %) as an off-white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.16(s, 1H, 5-ArH), 7.09(s, 2H, 4′ and 5′-ArH), 6.88(d, 2H, 1′ and 8′-ArH), 6.84(d, 2H, 2′ and 7′-ArH), 2.90(s, 4H, -CH_2CH_2-), 1.37(s, 18H, -tBuH). MALDI-TOF MS, m/z: 710.51, 613.34 (Calcd: 710.51). FT-IR(KBr), υ/cm^{-1}: 3442, 2975, 1784, 1757, 1612, 1496, 1424, 1371, 1157, 1116, 1028, 993, 795, 651.

Synthesis of 2′, 7′, 4, 7-tetrachloro-dipivaloyl-6-carboxyfluorescein N-hydroxysuccinimidyl ester **(4b)**

Compound **(4b)** was prepared from compound **3b** according to the procedure described above. Yield, 81.5 %, white solid, ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.15(s, 2H), 7.13(s, 1H), 6.93(s, 2H), 2.90(s, 4H), 1.45(s, 18H). FT-IR(KBr), υ/cm^{-1}: 3513, 2977, 1744, 1479, 1408, 1092. Anal. Calcd. for $C_{35}H_{27}NO_{11}Cl_4$: C 53.93, H 3.49, N 1.80; Found: C 54.13, H 3.69, N 1.90.

Synthesis of 4′, 5′, 4, 7-tetrachloro-dipivaloyl-6-carboxyfluorescein N-hydroxysuccinimidyl ester **(4c)**

Compound **(4c)** was prepared from compound **3c** according to the procedure described above. Yield, 82.3 %, white solid, ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.27(s, 1H), 6.96(d, 2H), 6.80 (d, 2H), 2.90(s, 4H), 1.42(s, 18H). FT-IR(KBr), υ/cm^{-1}: 3513, 2977, 1744, 1479, 1408, 1092. Anal. Calcd. for $C_{35}H_{27}NO_{11}Cl_4$: C 53.93, H 3.49, N 1.80; Found: C 54.03, H 3.23, N 1.74.

Synthesis of 2′, 4′, 5′, 7′, 4, 7-hexachloro-dipivaloyl-6-carboxyfluorescein N-hydroxysuccinimidyl ester **(4d)**

Compound **(4d)** was prepared from compound **3d** according to the procedure described above. Yield, 85 %.^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.29(s, 1H, 5-ArH), 6.88(s, 2H, 1′ and 8′-ArH), 2.94(t, 4H, -CH_2CH_2-), 1.45(s, 18H, t-BuH). MALDI-TOF MS, m/z: 848.25, 751.86 (Calcd: 848.29). FT-IR(KBr), υ/cm^{-1}: 3365, 2947, 1780, 1739, 1647, 1436, 1373, 1210, 1065, 647.

Synthesis of 4, 7-dichloro-dipivaloyl-6-(3-carboxypropylaminocarbonyl) fluorescein **(5at)**

To compound **4a** (4.62 g, 6.5 mmol) in 50 mL anhydrous dichloromethane was added dropwise a solution of 4-aminobutenoic acid (6.5×1.2 mmol, 0.80 g) in 20 mL CH_2Cl_2 under stirring. The resultant mixture was stirred at room temperature for 1–2 h. TLC ($CHCl_3$ / MeOH = 10:1) indicated the completion of the reaction. The solvent was removed under reduced pressure, and the residual crude product was purified by flash column chromatography on silica gel eluted with $CHCl_3$/EtOAc = 4/1, 3/1, 2/1 to yield compound **5at** (3.84, 84.5 %)as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.06(s, 2H, 4′ and 5′-ArH), 6.86(d, 2H, 1′ and 8′-ArH), 6.81(d, 2H, 2′ and 7′-ArH), 7.76(s, 1H, 5-ArH), 3.55(t, 2H, -CH_2-), 2.49(t, 2H, -CH_2-), 1.95–1.99(m, 2H, -CH_2-), 1.36(s, 18H, -tBuH). FT-IR(KBr), υ/cm^{-1}: 3380, 2974, 2935, 2875, 1780, 1758, 1652, 1611, 1547, 1496, 1423, 1373, 1218, 1157, 1115, 1027, 992, 896.

Synthesis of 4, 7-dichloro-dipivaloyl-6-(2-carboxyethylaminocarbonyl) fluorescein **(5ap)**

Compound **(5ap)** was prepared from compound **4a** and 3-aminopropanoic acid by the procedure utilized for compound **5 at.** Compound **5ap** (3.69, 83 %) was obtained as white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.07(s, 2H, 4′ and 5′-ArH), 6.87(d, 2H, 1′ and 8′-ArH), 6.82(d, 2H, 2′ and 7′-ArH), 7.79(s, 1H, 5-ArH), 3.78(t, 2H, -CH_2-), 2.78(t, 2H, -CH_2-),1.36(s, 18H, -tBuH). FT-IR(KBr), υ/cm^{-1}: 2976, 1758, 1612, 1495, 1424, 1157, 1115, 730.

Synthesis of 2′, 7′, 4, 7-tetrachloro-dipivaloyl-6-(3-carboxypropylaminocarbonyl) fluorescein **(5bt)**

Compound **(5bt)** was prepared from compound **4b** and 4-aminobutenoic acid by the procedure utilized for compound **5at.** Compound **5bt** (4.09 g, 82 %) was obtained as white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.05(s, 2H, 4′ and 5′-ArH), 6.95(d, 2H, 1′ and 8′-ArH), 7.72(s, 1H, 5-ArH), 3.47(t, 2H, -CH_2-), 2.52(t, 2H, -CH_2-), 1.20–1.25(m, 2H, -CH_2-), 1.35(s, 18H, -tBuH). FT-IR(KBr), υ/cm^{-1}: 3417, 2975, 2935, 2874, 1786, 1609, 1573, 1477, 1409, 1201, 1168, 1093, 1028, 896, 685.

Synthesis of 2′, 7′, 4, 7-tetrachloro-dipivaloyl-6-(2-carboxyethylaminocarbonyl) fluorescein **(5bp)**

Compound **(5bp)** was prepared from compound **4b** and 3-aminopropanoic acid by the procedure utilized for compound **5at.** Compound **5bp**, white solid, yield, 86 %, ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.13 (s, 2H, 4′ and 5′-ArH), 6.93 (d, 2H, 1′ and 8′-ArH), 7.84(s, 1H, 5-ArH), 3.78(t, 2H, -CH_2-), 2.77(t, 2H, -CH_2-), 1.39(s, 18H, -tBuH). FT-IR(KBr), υ/cm^{-1}: 3132, 2978, 1767, 1705, 1477, 1409, 1091, 614.

Synthesis of 4′, 5′, 4, 7-tetrachloro-dipivaloyl-6-(3-carboxypropylaminocarbonyl) fluorescein **(5ct)**

Compound **(5ct)** was prepared from compound **4c** and 4-aminobutenoic acid by the procedure utilized for compound **5at.** Compound **5ct**, white solid, yield, 83.4 %, ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.84(s, 2H, 2′ and 7′-ArH), 6.94(d, 2H, 1′ and 8′-ArH), 7.77(s, 1H, 5-ArH), 3.41(t, 2H, -CH_2-), 2.44(t, 2H, -CH_2-), 1.85–1.87(m, 2H, -CH_2-), 1.41(s, 18H, -tBuH). FT-IR(KBr), υ/cm^{-1}: 3386, 3215, 2975, 2875, 1786, 1609, 1641, 1567, 1478, 1425, 1212, 1092, 1058, 881, 728.

Synthesis of 4′, 5′, 4, 7-tetrachloro-dipivaloyl-6-(2-carboxyethylaminocarbonyl) fluorescein **(5cp)**

Compound **(5cp)** was prepared from compound **4c** and 3-aminopropanoic acid by the procedure utilized for compound **5at.** Compound **5cp**, white solid, yield, 84.2 %, ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.82(s, 2H, 2′ and 7′-ArH), 6.93(d, 2H, 1′ and 8′-ArH), 7.82(s, 1H, 5-ArH), 3.78(t, 2H, -CH_2-), 2.80(t, 2H, -CH_2-), 1.41(s, 18H, -tBuH). FT-IR(KBr), υ/cm^{-1}: 3424, 2977, 1767, 1600, 1477, 1425, 1398, 1096, 1052, 666.

Synthesis of 2′, 4′, 5′, 7′, 4, 7-hexachloro-dipivaloyl-6-(3-carboxypropylaminocarbonyl) fluorescein **(5dt)**

Compound **(5dt)** was prepared from compound **4d** and 4-aminobutenoic acid by the procedure utilized for compound **5at.** Compound **5dt**, white solid, yield, 80 %, ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.87(s, 2H, 1′ and 8′ -ArH), 7.83(s, 1H, 5-ArH), 3.57(t, 2H, -CH_2-), 1.96–2.00(m, 2H, -CH_2-), 2.53(t, 2H, -CH_2-), 1.44(s, 18H, -tBuH). FT-IR(KBr), υ/cm^{-1}: 3371, 2977, 1778, 1657, 1547, 1454, 1424, 1373, 1211, 1079, 1026, 754. Anal. Calcd for $C_{35}H_{29}Cl_6NO_{10}$: C 50.26, H 3.50, N 1.67; Found: C 50.28, H 3.51, N 1.66.

Synthesis of 2′, 4′, 5′, 7′, 4, 7-hexachloro-dipivaloyl-6-(2-carboxyethylaminocarbonyl) fluorescein **(5dp)**

Compound **(5dp)** was prepared from compound **4d** and 3-aminopropanoic acid by the procedure utilized for compound **5at.** Compound **5dp**, yield, 84.2 %, ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.89(s, 2H, 1′ and 8′ -ArH), 7.87(s, 1H, 5-ArH), 3.82(t, 2H, -CH_2-), 2.79(t, 2H, -CH_2-), 1.45(s, 18H, -tBuH). FT-IR(KBr), υ/cm^{-1}: 2976, 1775, 1453, 1426, 1261, 1211, 1081, 874.

Compound 4, 7-dichloro -6-(3-carboxypropylaminocarbonyl) fluorescein **(6at)**

Compound **5at** (4.19 g, 6 mmol) was dissolved in 50 mL of CH_2Cl_2. To the solution was added 10 mL of NH_4OH (28 %), and the mixture was stirred for 2 h. The reaction mixture was diluted with 60 mL of water. The water layer was acidified with 10 % HCl to pH 2. The resulting precipitate was collected, washed with cold water (3×15 mL), and dried in vacuum. **6at** as an orange solid was obtained in 78 % yield (2.48 g). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 10.19(s, 1H, -COOH), 6.77(s, 2H, 4′ and 5′-ArH), 6.67(d, 2H, 1′ and 8′-ArH), 6.57(d, 2H, 2′ and 7′-ArH), 7.92(s, 1H, 5-ArH), 3.29(t, 2H, -CH_2-), 2.32(t, 2H, -CH_2-), 1.72–1.79(m, 2H, -CH_2-). MALDI-TOF MS, m/z: 530.87 (Calcd:530.31). FT-IR(KBr), υ/cm^{-1}: 3379, 2960, 1732, 1687, 1609, 1560, 1505, 1449, 1377, 1224, 1177, 1138, 1112, 992, 923, 844, 664.

Compound 4, 7-dichloro -6-(2-carboxyethylaminocarbonyl) fluorescein **(6ap)**

Starting from compound **5ap** and using a similar procedure as described above, compound **(6ap)** was obtained in 75 % yield (2.32 g). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 10.21(s, H, -COOH), 6.66(s, 2H, 4′ and 5′-ArH), 6.76(d, 2H, 1′ and 8′-ArH), 6.55(d, 2H, 2′ and 7′-ArH), 7.84(s, 1H, 5-ArH), 3.40(t, 2H, -CH_2-), 2.54(t, 2H, -CH_2-). MALDI-TOF MS, m/z: 516.92 (Calcd:516.28). FT-IR(KBr), υ/cm^{-1}: 3343, 3080, 1741, 1608, 1506, 1450, 1376, 1227, 1180, 1140, 1113, 994, 930, 846, 813, 665.

Compound 2′, 7′, 4, 7-tetrachloro-6-(3-carboxypropylaminocarbonyl) fluorescein **(6bt)**

Starting from compound **5bt** and using a similar procedure as described above, compound **(6bt)** was obtained in 83 % yield (2.98 g). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.11(s, 1H, -COOH), 7.21(s, 2H, 4′ and 5′-ArH), 6.86(s, 2H, 1′ and 8′-ArH), 8.04(s, 1H, 5-ArH), 3.17(t, 2H, -CH_2-), 2.23(t, 2H, -CH_2-), 1.64–1.74(m, 2H, -CH_2-). MALDI-TOF MS, m/z: 600.47 (Calcd: 599.2). FT-IR(KBr), υ/cm^{-1}: 3419, 3302, 1752, 1705, 1656, 1602, 1558, 1432, 1310, 1231, 1179, 1131, 1056, 1032, 918, 810, 725, 663.

Compound 2′, 7′, 4, 7-tetrachloro-6-(2-carboxyethylaminocarbonyl) fluorescein **(6bp)**

Starting from compound **5bp** and using a similar procedure as described above, compound **(6bp)** was obtained in 77 % yield (2.70 g). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.13(s, 1H, -COOH), 7.23(s, 2H, 4′ and 5′-ArH), 7.10(s, 2H, 1′ and 8′-ArH), 8.12(s, 1H, 5-ArH), 3.62(t, 2H, -CH_2-), 2.56(t, 2H, -CH_2-). MALDI-TOF MS, m/z: 585.60 (Calcd: 585.17). FT-IR(KBr), υ/cm^{-1}: 3420, 1740, 1606, 1489, 1433, 1376, 1267, 1171, 1138, 953, 867, 690.

Compound 4′, 5′, 4, 7-tetrachloro-6-(3-carboxypropylaminocarbonyl) fluorescein **(6ct)**

Starting from compound **5ct** and using a similar procedure as described above, compound **(6ct)** was obtained in 82 % yield (2.94 g). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.08(s, H, -COOH), 6.84(s, 2H, 2′ and 7′ -ArH), 6.87(s, 2H, 1′ and 8′-ArH), 7.90(s, 1H, 5-ArH), 3.19(t, 2H, -CH_2-), 2.22(t, 2H, -CH_2-), 1.62–1.69(m, 2H, -CH_2-). MALDI-TOF MS, m/z: 599.40 (Calcd: 599.2). FT-IR(KBr), υ/cm^{-1}: 3427, 3065, 1737, 1629, 1610, 1503, 1432, 1265, 1178, 1121, 1046, 876, 744.

Compound 4′, 5′, 4, 7-tetrachloro-6-(2-carboxyethylaminocarbonyl) fluorescein **(6cp)**

Starting from compound **5cp** and using a similar procedure as described above, compound **(6cp)** was obtained in 78 % yield (2.74 g). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 10.99(s, 1H, -COOH), 6.83(s, 2H, 2′ and 7′ -ArH), 6.92(s, 2H, 1′ and 8′-ArH), 7.89(s, 1H, 5-ArH), 3.46(t, 2H, -CH_2-), 2.48(t, 2H, -CH_2-), 1.62–1.69(m, 2H, -CH_2-). MALDI-TOF MS, m/z: 588.44 (Calcd: 585.17).

Compound 2′, 4′, 5′, 7′, 4, 7-hexachloro-6-(3-carboxypropylaminocarbonyl) fluorescein **(6dt)**

Starting from compound **5dt** and using a similar procedure as described above, compound **(6dt)** was obtained in 76 % yield (3.04 g). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.11(s, 2H, 1′ and 8′ -ArH), 7.95(s, 1H, 5-ArH), 3.45(t, 2H, -CH_2-), 1.72-1.80(m, 2H, -CH_2-), 2.35(t, 2H, -CH_2-). MALDI-TOF MS, m/z: 669.00, (Calcd: 668.09). FT-IR(KBr), υ/cm^{-1}: 3579, 3333, 1772, 1646, 1559, 1436, 1198, 1150, 1092, 749. Anal. Calcd for $C_{25}H_{13}Cl_6NO_8$: C 44.94, H 1.96, N 2.10; Found: C 44.95, H 1.95, N 2.09.

Compound 2′, 4′, 5′, 7′, 4, 7-hexachloro-6-(2-carboxyethylaminocarbonyl) fluorescein **(6dp)**

Starting from compound **5dp** and using a similar procedure as described above, compound **(6dp)** was obtained in 76 % yield (2.98 g). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 10.99(s, 1H, -COOH), 7.26(s, 2H, 1′ and 8′ -ArH), 7.87(s, 1H, 5-ArH), 3.47(t, 2H, -CH_2-), 2.54(t, 2H, -CH_2-). MALDI-TOF MS, m/z: 654.28, (Calcd: 654.06). FT-IR(KBr), υ/cm^{-1}: 3410, 1769, 1642, 1476, 1433, 1213, 1141, 101, 863.

Synthesis of 4-(4, 7-dichlorofluorescein-6-carboxamido) -butanoic acid succinimidyl ester **(7at)**

To compound **6at** (2.65 g, 5 mmol) in 50 mL anhydrous dichloromethane was added anhydrous pyridine (16 mL) and N-hydroxysuccinimidyl trifluoroacetate (NHS-TFA) (8.44 g, 40 mmol) under vigorous stirring. The resultant mixture was stirred at room temperature for 6 h. TLC ($CHCl_3$/MeOH=5:1) was used to confirm the completion of the reaction. The mixture was washed with 4 % HCl (2×100 mL) and saturated saline (2×50 mL), dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure, and residue was dried in vacuum to yield compound **7at** as a yellow solid in 80 % yield (2.51 g). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 10.19(s, 1H, -COOH), 6,78(s, 2H, 4′ and 5′-ArH), 6.67(d, 2H, 1′ and 8′-ArH), 6.57(d, 2H, 2′ and 7′-ArH), 7.99(s, 1H, 5-ArH), 3.34(t, 2H, -CH_2-), 2.59(t, 2H, -CH_2-), 1.87–1.90(m, 2H, -CH_2-), 2.82(t, 4H, -CH_2CH_2-). MALDI-TOF MS, m/z: 627.33, (Calcd: 627.38). FT-IR(KBr), υ/cm^{-1}: 3408, 2938, 1812, 1768, 1734, 1613, 1507, 1449, 1376, 1216, 1181, 1070, 994, 668.

Compound 3-(4, 7-dichlorofluorescein-6-carboxamido) -propanoic acid succinimidyl ester **(7ap)**

Starting from compound **6ap** and using a similar procedure as described above, compound (**7ap)** was obtained as a yellow solid in 78 % yield (2.39 g). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 10.20(s, 1H, -COOH), 6,78(s, 2H, 4′ and 5′-ArH), 6.67(d, 2H, 1′ and 8′-ArH), 6.56(d, 2H, 2′ and 7′-ArH), 7.90(s, 1H, 5-ArH), 3.59(t, 2H, -CH_2-), 3.05 (t, 2H, -CH_2-), 2.82(t, 4H, -CH_2CH_2-). MALDI-TOF MS, m/z: 613.54, (Calcd: 613.36). FT-IR(KBr), υ/cm^{-1}: 3383, 1814, 1769, 1735, 1612, 1507, 1450, 1375, 1214, 1181, 1072, 993, 850.

Compound 4-(2′, 7′, 4, 7-tetrachlorofluorescein-6-carboxamido)-butanoic acid succinimidyl ester **(7bt)**

Starting from compound **6bt** and using a similar procedure as described above, compound (**7bt**) was obtained as a deep orange solid in 75 % yield (2.61 g). ^{1}HNMR (400 MHz, DMSO-d_6) δ: 11.03(s, 1H, -COOH), 6.18(s, 2H, 4′ and 5′-ArH), 6.87(d, 2H, 1′ and 8′-ArH), 7.92(s, 1H, 5-ArH), 3.42(t, 2H, -CH_2-), 2.59(t, 2H, -CH_2-), 1.81–1.84(m, 2H, -CH_2-), 2.81(t, 4H, -CH_2CH_2-). MALDI-TOF MS, m/z: 697.42, (Calcd: 696.27). FT-IR (KBr), υ/cm^{-1}: 3429, 2929, 1812, 1764, 1734, 1630, 1608, 1530, 1435, 1379, 1204, 1141, 1066, 960, 681.

Compound 3-(2′, 7′, 4, 7-tetrachlorofluorescein-6-carboxamido)-propanoic acid succinimidyl ester **(7bp)**

Starting from compound **6bp** and using a similar procedure as described above, compound (**7bp)** was obtained as a deep orange solid in 75 % yield (2.56 g). ^{1}HNMR (400 MHz, DMSO-d_6) δ: 11.14(s, H, -COOH), 7.10(s, 2H, 4′ and 5′-ArH), 6.88(d, 2H, 1′ and 8′-ArH), 7.89(s, 1H, 5-ArH), 3.60(t, 2H, -CH_2-), 3.04(t, 2H, -CH_2-), 2.82(t, 4H, -CH_2CH_2-). MALDI-TOF MS, m/z: 683.08, (Calcd: 682.25). FT-IR (KBr), υ/cm^{-1}: 3378, 2939, 1814, 1772, 1735, 1612, 1506, 1450, 1376, 1215, 1179, 1067, 850, 650.

Compound 4-(4′, 5′, 4, 7-tetrachlorofluorescein-6-carboxamido)-butanoic acid succinimidyl ester **(7ct)**

Starting from compound **6ct** and using a similar procedure as described above, compound (**7ct**) was obtained as a salmon pink solid in 78 % yield (2.72 g). ^{1}HNMR (400 MHz, DMSO-d_6) δ: 11.01(s, 1H, -COOH), 6.87(s, 2H, 2′ and 7′-ArH), 6.82(d, 2H, 1′ and 8′-ArH), 7.96(s, 1H, 5-ArH), 3.26(t, 2H, -CH_2-), 2.72(t, 2H, -CH_2-), 1.79–1.83(m, 2H, -CH_2-), 2.80(t, 4H, -CH_2CH_2-). MALDI-TOF MS, m/z: 697.59, (Calcd: 696.27). FT-IR(KBr), υ/cm^{-1}: 3410, 3310, 1739, 1658, 1601, 1563, 1433, 1371, 1205, 1132, 1059, 645.

Compound 3-(4′, 5′, 4, 7-tetrachlorofluorescein-6-carboxamido)-propanoic acid succinimidyl ester **(7cp)**

Starting from compound **6cp** and using a similar procedure as described above, compound (**7cp)** was obtained as a salmon pink solid in 76 % yield (2.59 g). ^{1}HNMR (400 MHz, DMSO-d_6) δ: 11.04(s, 1H, -COOH), 7.17(d, 2H, 2′ and 7′-ArH), 6.92(d, 2H, 1′ and 8′-ArH), 7.95(s, 1H, 5-ArH), 3.45(t, 2H, -CH_2-), 2.60(t, 2H, -CH_2-), 2.81(t, 4H, -CH_2CH_2-). MALDI-TOF MS, m/z: 683.65, (Calcd: 682.25). FT-IR(KBr), υ/cm^{-1}: 3401, 1773, 1738, 1708, 1605, 1434, 1217, 1060.

Compound 4-(2′, 4′, 5′, 7′, 4, 7-hexachlorofluorescein-6-carboxamido)-butanoic acid succinimidyl ester **(7dt)**

Starting from compound **6dt** and using a similar procedure as described above, compound (**7dt**) was obtained as a red solid in 80 % yield (3.06 g). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.22(s, 1H, -COOH), 8.78(s, 1H, ArOH), 8.00(s, 1H, 5-ArH), 7.29(s, 2H, 1′ and 8′ -ArH), 3.44(t, 2H, -CH_2-), 1.88–1.91(m, 2H, -CH_2-), 2.73(t, 2H, -CH_2-), 2.82(t, 4H, -CH_2CH_2-). MALDI-TOF MS, m/z: 766.36, (Calcd: 765.16). FT-IR(KBr), υ/cm^{-1}: 3318, 2981, 1785, 1732, 1649, 1600, 1430, 1207, 1064, 747, 868, 649.

Compound 3-(2′, 4′, 5′, 7′, 4, 7-hexachlorofluorescein-6-carboxamido)-propanoic acid succinimidyl ester **(7dp)**

Starting from compound **6dp** and using a similar procedure as described above, compound (**7dp)** was obtained as a red solid in 79 % yield (2.97 g). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.21(s, 1H, -COOH), 8.73(s, 1H, ArOH), 7.90(s, 1H, 5-

ArH), 7.29(s, 2H, 1′ and 8′ -ArH), 3.51(t, 2H, -CH_2-), 2.59(t, 2H, -CH_2-), 2.81(t, 4H, -CH_2CH_2-). MALDI-TOF MS, m/z: 751.65, (Calcd: 751.14). FT-IR (KBr), υ/cm^{-1}: 3339, 1816, 1707, 1430, 1216, 1080, 1001, 867, 813.

Preparation of Working Solution

The 1.25×10^{-3} mol L^{-1} sample stock solutions were prepared by dissolving the sample in dimethyl sulfoxide (DMSO) and further diluted with distilled water to result in 1.25×10^{-5} mol L^{-1} stock solutions of sample containing 1 % DMSO.

Working solutions, which were used to study spectroscopic properties and measure fluorescence quantum yields, were prepared by diluting with 0.1 M NaOH. The solution concentration of these solutions was 5.0×10^{-7} mol L^{-1}. All working solutions were prepared immediately before the experiment.

Fluorescence Properties and Fluorescence Quantum Yields

Fluorescence spectroscopic studies were performed on a Hitachi F-4500 spectrofluorimeter. The slit width was 10 nm for excitation and 5 nm for emission. The excitation wavelength was 470 nm. For determination of the fluorescence quantum yields, fluorescein in 0.1 M NaOH (Φ_F=0.79) was used as a standard [19]. Fluorescence quantum yields were obtained from multiple measurements (N=3) with the following equation [20–24]

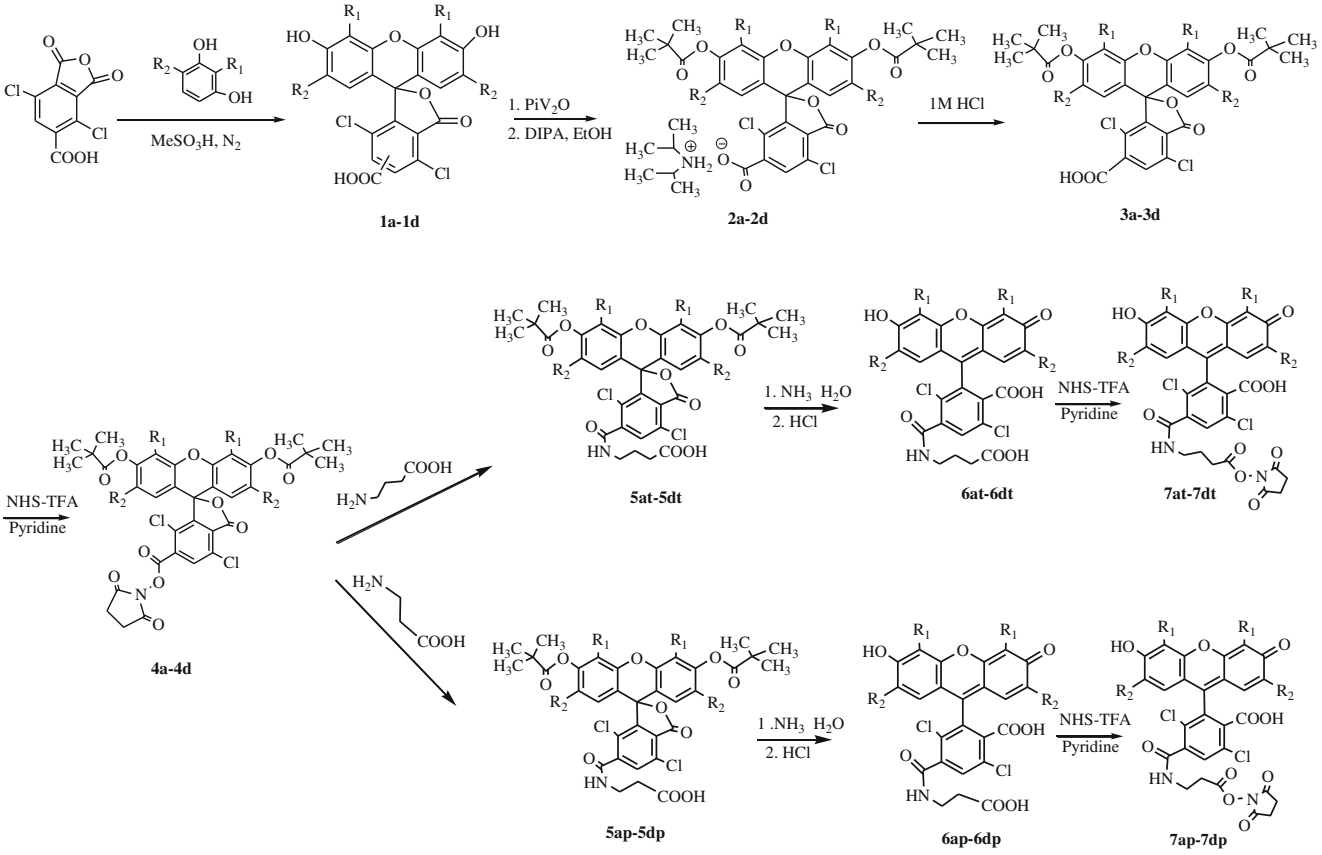
$$\phi^{(\text{sample})} = \phi^{(\text{standard})}\frac{Abs^{(\text{standard})}\sum\left[F^{(\text{sample})}\right]}{Abs^{(\text{sample})}\sum\left[F^{(\text{standard})}\right]}$$

Where ΣF denotes integrated fluorescence intensity which is the area of the fluorescence spectrum from the fully corrected fluorescence spectra. In the equation, *Abs* denotes absorbance at the excitation wavelength (470 nm). The *Abs* was obtained from the absorption spectra.

Results and Discussion

Synthesis and Chemical Properties of Probes

These chlorinated fluorescent probes were synthesized according to the synthetic procedure shown in Scheme 1.



1a, 2a, 3a, 4a, 5at, 6at, 7at, 5ap, 6ap, 7ap: R_1=H, R_2=H
1b, 2b, 3b, 4b, 5bt, 6bt, 7bt, 5bp, 6bp, 7bp: R_1=H, R_2=Cl
1c, 2c, 3c, 4c, 5ct, 6ct, 7ct, 5cp, 6cp, 7cp: R_1=Cl, R_2=H
1d, 2d, 3d, 4d, 5dt, 6dt, 7dt, 5dp, 6dp, 7dp: R_1=Cl, R_2=Cl

Scheme 1 Synthesis of eight novel fluorescent proteins-labeling probes

Table 1 Spectral properties of eight probes

Compounds	7at	7ap	7bt	7bp	7ct	7cp	7dt	7dp
λ_{em}(nm)	524	524	534	534	539	539	550	550
λ_{ex}(nm)	508	508	521	521	522	522	536	536
λ_{ab}(nm)	508	508	521	521	522	522	536	536
Stokes shift(nm)	16	16	13	13	17	17	14	14
Abs(pH=13)	0.067	0.044	0.022	0.041	0.074	0.086	0.051	0.053
$\varepsilon(M^{-1}\ cm^{-1}\times10^4)$	8.43	8.33	14.99	14.23	8.05	8.10	15.66	15.92
Φ_F[a](pH=13)	0.72	0.72	0.71	0.71	0.68	0.68	0.65	0.65

[a] Fluorescent was used as a standard (Φ_F=0.79 in 0.1 M NaOH), see reference [19]

There were two potential approaches to the synthesis of chlorinated fluoresceins. One method was chlorination of the building blocks of fluorescein and then synthesis of chlorinated fluoresceins; the other was the directed chlorination of fluorescein. There were several active positions on the fluorescein that are readily available for direct chlorination by electrophilic chlorination reagents. However, the isolation of pure products from chlorinated fluoresceins was a very difficult problem. Therefore, we selected the first method in this work.

In this paper, chlorinated fluoresceins were synthesized by condensation of the appropriate chlorinated resorcinol and 3, 6-dichloro-4-carboxyphthalic anhydride in the presence of methanesulfonic acid. Methanesulfonic acid as a catalyzer was reported in many references. It was found that methanesulfonic acid served as both a Lewis acid catalyst and a suitable solvent in the dye-forming reaction, giving a good procedure with higher yields of products under milder conditions.[5] The high yield was obtained by gradual increase of temperature. The better procedure was that the reaction mixture was warmed and stirred under nitrogen at 50 °C for 4 h then at 110 °C for 24 h.

Carboxyfluorescein was prepared and commonly used as mixture of two isomers, namely 5- and 6-carboxyfluorescein. A few reports suggested that two isomers have some differences in behavior and subsequent binding assays [25, 26]. These two isomers had almost identical spectroscopic properties, making a direct comparison of lectin-binding properties possible. Working with single isomers also significantly simplified chromatographic separations and subsequent characterizations by NMR [27].

The literature[2, 28] reported that the 5(6)-isomers can be separated at this point by selective crystallization of the 6-isomer as its diisopropylamine salt from ethanol, and the 5-isomer can be recovered from the supernatant and recrystallized from nitromethane. We successfully separated the 6-isomer as its diisopropylamine salt from ethanol. However, we were never successful in obtaining the 5-isomer in pure form via this route.

When carboxyfluorescein was refluxed in pivalic anhydride, make sure it was completely converted to its dipivalae. Reflux time should be more than two hours and the pivalic anhydride should be overdose. After diisopropylamine was added, the mixture should be shaken several times and then

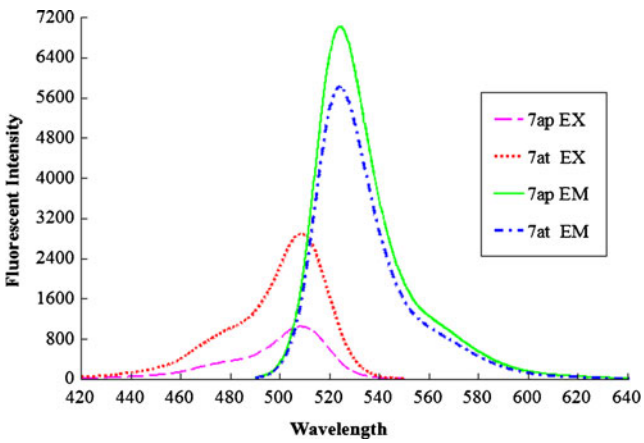


Fig. 1 Excitation and emission spectra of compounds **7ap** and **7at**. Concentration of all solutions was 5.0×10^{-7} mol L^{-1}

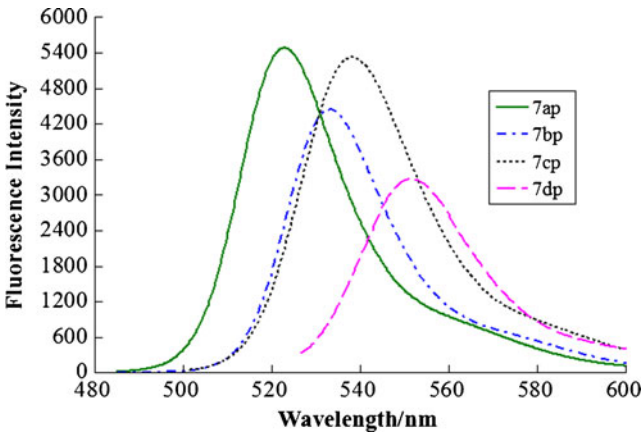


Fig. 2 Fluorescent emission spectra of compounds **7ap**, **7bp**, **7cp** and **7dp**

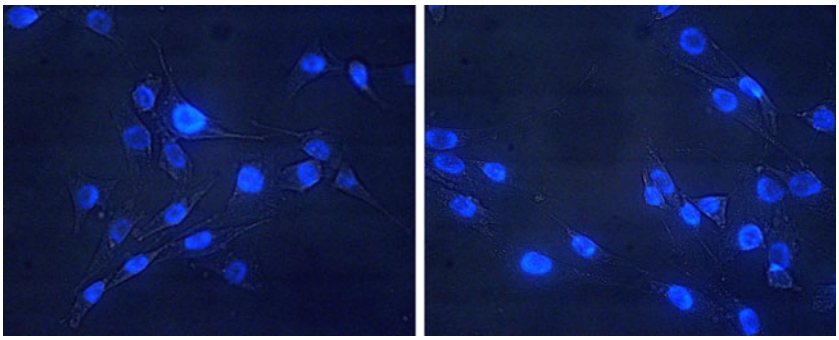


Fig. 3 Fluorescent microscopic images of the living MC3T3-E1 cells directly labeled by **7ap** in the blue channels

the mixture was cooed to −20 °C in freezer overnight. Small-scale test showed that the solubility of 5-isomer carboxyfluorescein diisopropylamine salt is much better in ethanol at room temperature than 6-isomer. If the 6-isomer carboxyfluorescein diisopropylamine salt is not very pure, pure 6-isomer can be afforded by the treatment of it with ethanol (sonicated for 10 min) several times.

N-hydroxysuccinimide (NHS) esters are among the most commonly used reagents for modification of amine groups. These reagents have intermediate reactivity toward amines, with high selectivity toward aliphatic amines. However, their reaction rate with aromatic amines, phenols (tyrosine), alcohols, and histidine is relatively low [29]. In this work, our chlorinated fluorescent probes were designed by introducing N-hydroxysuccinimide as reactive group, which is the most amine-selective and stable reactive group for labeling proteins, peptides, and other amines. The aliphatic amide products are very stable, which are formed by reacting NHS ester of the probe with the terminal amine group. Although the NHS esters are slowly hydrolyzed by water, they are stable to storage if kept well desiccated. Virtually any molecule that contains a carboxylic acid or that can be chemically modified to contain a carboxylic acid can be converted into its NHS ester, making these reagents among the most powerful protein-modification reagents available.

In order to facilitate and improve conjugations, we have added a spacer linker between the chlorinated fluorophore and succinimidyl ester group. The spacer linker was designed to reduce potential interactions between the probe dye and the protein. 4-Aminobutanoic acid or 3-amiopropanoic acid was used as a spacer linker. This allowed us to prepare succinimidyl ester derivatives, the most amine-selective and stable reactive group for labeling proteins.

Spectral Properties of Eight Probes

The wavelength of fluorescence emission maxima, the wavelength of fluorescence excitation maxima, the wavelength of absorption maxima, Stokes shift, fluorescence quantum yields and molar extinction coefficient are summarized in Table 1 for these probes. All of the compounds showed in Table 1 had excitation maxima in the range 508–536 nm, and their molar extinction coefficients were high ((8.05–15.92) × 10^4 M^{-1} cm^{-1}). They exhibited interesting fluorescence properties at room temperature. Their fluorescence maxima were located at 524–550 nm. Fluorescent quantum yields for these compounds were all reasonably high for the fluorescent probes.

The chlorine fluorescein structure can be divided into two parts, the benzene moiety and the fluorophore [30]. Compounds **7ap** and **7at**, which have the same fluorophore but different benzene moieties, almost had the same Ex / Em maximum (See Table 1 and Fig. 1). Compounds **7bp** and **7bt** had the same phenomenon. From Fig. 2, the chlorinated fluorescent probes were found to shift the emission spectra towards long

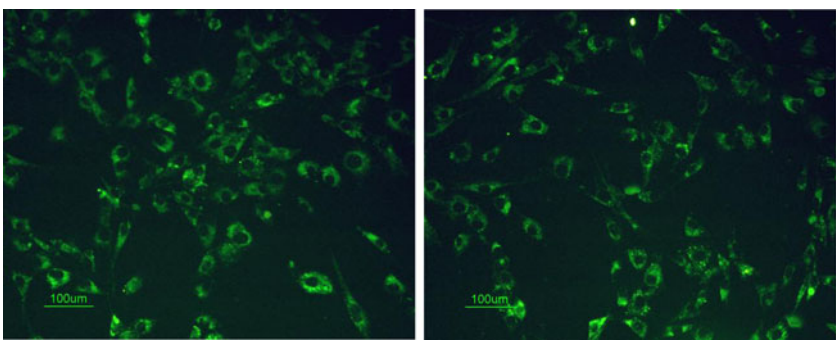


Fig. 4 Fluorescent microscopic images of fixed MC3T3-E1 cells directly labeled by **7ct** in the green channels

wavelength, and shift of the spectra toward long wavelength increased with increase in chlorine. For example, 3-(2′, 4′, 5′, 7′, 4, 7-hexachlorofluorescein-6-carboxamido)-propanoic acid succinimidyl ester (**7dp)** with four chlorine substitutions on fluorophore had emission maxima at 550 nm, however, 3-(4, 7-dichlorofluorescein-6-carboxamido)-propanoic acid succinimidyl ester (**7ap)** without chlorine atoms on fluorophore had emission maxima at 524 nm. A red shift of 26 nm was observed between compounds **7dp** and **7ap**. The reason for this phenomenon might be the electron-withdrawing ability of the chlorine substituted on fluorophore. The structure of fluorophore played an important role in fluorescent excitation and emission spectra. However, the benzene moiety almost had no effect on excitation and emission.

We chose compounds **7ap** and **7ct** for labeling experiments. Firstly, we incubated the living MC3T3-E1 cells grown on a coverslip with **7ap** at 25 °C for 45 min. Figure 3 showed that the probe is localized in the nucleus. This was attributed to the fact that the probe dye attached on the cell membrane was taken up by the MC3T3-E1 cells and transferred into the cell nucleus, while the cells maintain their characteristic nematomorphic morphology and well-spread pattern. These observations further indicated that the incubation of MC3T3-E1 cells with the fluorescent **7ap** causes almost no harmful effect to the essential activities of MC3T3-E1 cells, such as cell attachment and spread. This indicated that these chlorinate fluorescent dyes could be a favorable intercellular imaging probe with the ability of cell penetration. We also stained the chemical fixed MC3T3-E1 with the compound **7ct** (see Fig. 4). To our surprise, we found that the dye is localized in cytosol instead of nucleus. Certainly, under the circumstances the dye might be only conjugates to the cell surface.

In conclusion, we have synthesized eight novel chlorinated fluorescent proteins-labeling probes with a linker and reactive group in 7 steps. Thirty-two intermediate compounds were also prepared. To our knowledge, most of compounds have not been reported.

The spectral properties of the chlorinated fluoresceins were studied in detail. These fluorescent probes showed absorbance peaks at 508–536 nm and emission peaks at 524–550 nm with high quantum yields. We also confirmed that red shift of emission, 26 nm, occurs depending on the increase of chlorine atom.

The probes were used for fluorescence imaging of cells in order to investigate whether they can conjugate to cells. The fluorescence imaging of living cells showed that probes are localized in cell nucleus. However, the imaging of fixed cells showed that probes are localized in cytosol of chemically fixed cells. These probes will be useful reagents for the preparation of stable fluorescent conjugates. If these probes are conjugated to anti-IgG antibodies, they can also be used in immunofluorescent histochemistry.

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