Bioorganic & Medicinal Chemistry Letters 19 (2009) 2957–2959

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis and biological applications of two novel fluorescent proteins-labeling probes

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ARTICLE INFO

Article history:
Received 19 September 2008
Revised 15 March 2009
Accepted 15 April 2009
Available online 18 April 2009

Keywords:
Fluorescent dye
Probe dye
Cell labeled
Synthesis

ABSTRACT

Two novel chlorinated fluoresceins 2′,4′,5′,7′-tetrachloro-6-(5-carboxypentyl)-4,7-dichloro fluorescein succinimidyl ester (**1G**) and 2′,4′,5′,7′-tetrachloro-6-(3-carboxypropyl)-4,7-dichlorofluorescein succinimidyl ester (**2G**) were synthesized as fluorescent probes for labeling proteins. Structures of target compounds and intermediates were determined via IR, MS, ^{1}H NMR and element analysis. The investigation in immunofluorescence histochemistry showed them had strong fluorescence, high photostability and good biocompatibility.

The carboxy-functionalized fluorescein[1] and rhodamine dyes have become increasingly important as conjugated fluorescent markers of biologically active compounds.[2] They are widely used in sensing applications owing to their brightness, high quantum yields, low-energy excitation and emission wavelengths, and biocompatibility.[3] Although these dyes share a common, 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 emission and absorbance maxima compared with fluorescein. The characteristic is very useful for multi-color imaging.

Two chlorinated fluoresceins 4,7,2′,7′-tetrachloro-6-(5-carboxypentyl)fluorescein and 4,7,4′,5′-tetrachloro-6-(5-carboxypentyl)fluorescein had been synthesized as fluorescent probes for labeling proteins by our group.[4] The research on chlorinated fluorescein is continued. Two novel chlorinated fluoresceins 2′,4′,5′,7′-tetrachloro-6-(5-carboxypentyl)-4,7-dichlorofluorescein succinimidyl ester (**1G**) and 2′,4′,5′,7′-tetrachloro-6-(3-carboxypropyl)-4,7-dichloro fluorescein succinimidyl ester (**2G**) will be reported in this Letter. These compounds were more photostable than the non-chlorinated fluoresceins.[5] In addition, the lower pK_a of chlorinated fluoresceins make their fluorescence essentially pH insensitive in the physiological pH range.[6] The fluorescence quenching was minimized by using the 6-aminohexanoic acid or 4-aminobutanoic acid as spacer linker. These characteristics make these novel chlorinated fluoresceins as available tools for a variety of biological applications.

The synthesis of compounds **1G** and **2G** were summarized in Scheme 1. The compounds 2,4-dichlororesorcinol and 3,5-dichloro-2,4-dihydroxybenzonic acid were condensed with methanesulfonic acid by the molar ratio of 2:1 to get the mixture of 5- and 6-isomer carboxy-functionalized fluoresceins(**A**) with excellent yields in Scheme 1. The other catalyzers such as $ZnCl_2$ and sulfuric acid can also be used for the condensation, but methanesulfonic acid gave a better yield. In order to separate the mixed 5-and 6-isomers, the mixture was heated in pivalic anhydride to give a crude mixture of chlorinated fluorescein dipivalates. When this mixture was dissolved in ethanol and treated with diisopropylamine, an insoluble 6-isomer diisopropylamine salt (**B**) selectively formed. Pure **B** (6-iosmer) can be obtained by recrystallization in the form of diisopropylamine salt using anhydrous ethanol as the solvent. The diisopropylamine salt **B** was converted to the carboxylic acid **C** by treatment with 1 M HCl. Structures and purities of **B** and **C** were confirmed by ^{1}H NMR, MOLDI-TOF MS and IR.[7]

Corresponding succinimidyl ester **D** was obtained by reaction of compound **B** with *N*-hydroxysuccinimidyl trifluoroacetate (NHS–TFA). Compound **D** which is highly reactive activity was easily reacted with 6-aminohexanoic acid to give compound **1E**. Column chromatography using 4:1 chloroform–ethyl acetate mixture as the eluent gave pure **1E** as a white solid. Compound **1E** was firstly treated with ammonia. Then, the reaction mixture was acidified with 1 M HCl to give a red solid **1F**. Compound **1F** was converted to its succinimidyl ester **1G**, which was used for our biological

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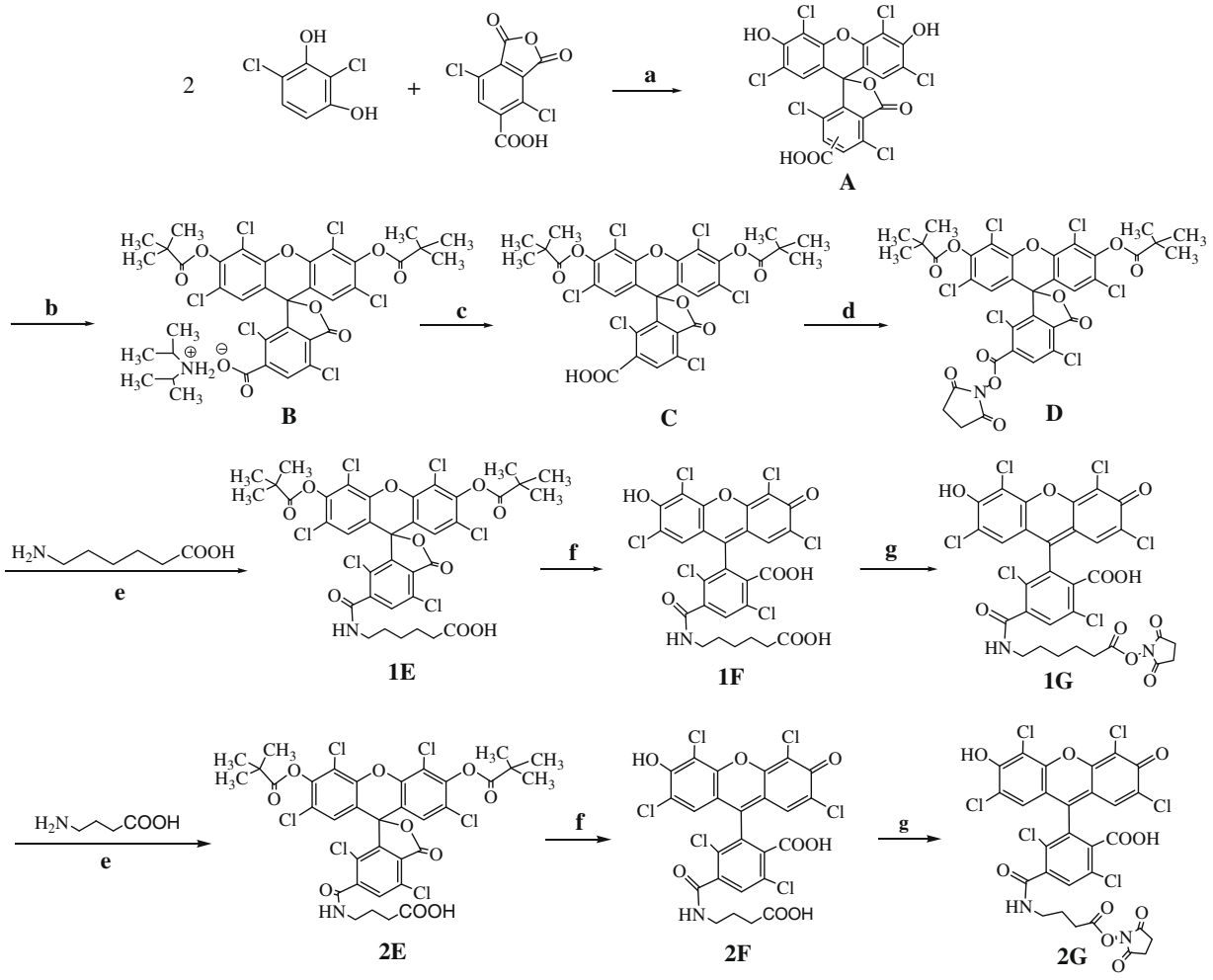
doi:10.1016/j.bmcl.2009.04.049



Scheme 1. Synthesis of two novel chlorinated fluorescein succinimidyl esters **1G** and **2G**. Reagents and conditions: (a) methyl sulfonic acid, N_2, 48 h, 100 °C; (b) 1. pivalic anhydride, reflux; 2. diisopropylamine, anhyd alcohol; **B** yield: 38% ; (c) 1 M HCl; **C** yield 93%; (d) NHS–TFA, pyridine, CH_2Cl_2 rt.; **D** yield 83%; (e) 6-aminohexanoic acid/4-aminobutanoic acid, CH_2Cl_2 rt. **1E** yield 63%, **2E** yield 67%; (f) (1) NH_3/H_2O; (2) 1 M HCl; **1F** yield 89%, **2F** yield 93%; (g) NHS–TFA, pyridine, CH_2Cl_2 rt; **1G** yield 78%, **2G** yield 82%.

applications. The compound **D** was reacted with 4-aminobutanoic acid to give compound **2E**. Use the same way above, we can get the compound **2G**.

Reactive esters, especially *N*-hydroxysuccinimide (NHS) esters, are among the most commonly used reagents for modification of amine groups. These novel chlorinated fluorescein succinimidyl esters are quite stable if they are properly stored. They have intermediate reactivity toward amines, with high selectivity toward aliphatic amines. Their reaction rate with aromatic amines, alcohols, phenols and histidine is relatively low. The excitation and emission spectrums of compound **1G** and **2G** were shown in Figures 1 and 2. From the spectrum we can see that they have the same maximum absorption wavelength of 535 nm and maximum emission wavelength of 550 nm. The large Stoke shift about 15 nm was shown. To our knowledge, compounds **B**, **C**, **D**, **1E** (**2E**), **1F** (**2F**) and **1G** (**2G**) have not been reported in the literature. Structures of **D**, **1E** (**2E**), **1F** (**2F**) and **1G** (**2G**) were also confirmed by ^{1}H NMR, MOLDI-TOF MS and IR.[8]

In an effort to facilitate and improve conjugations, a 6-aminohexanoic acid or a 4-aminobutanoic acid spacer was added

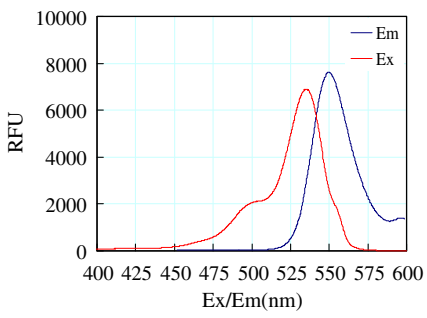


Figure 1. Excitation and emission spectrum of compound **1G** in 0.1 M NaOH.

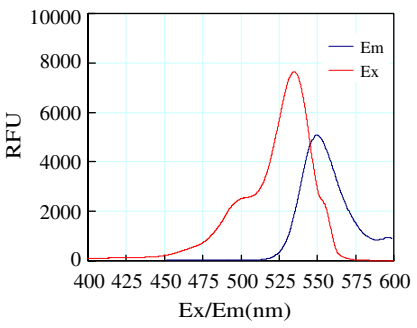


Figure 2. Excitation and emission spectrum of compound **2G** in 0.1 M NaOH.

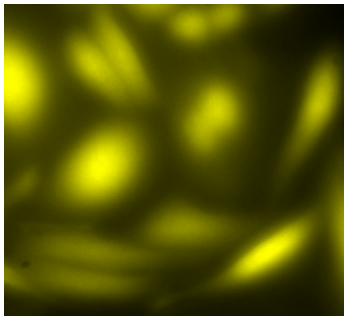


Figure 3. Fluorescence images of U2OS cells in 96-well Costar black plate were fixed with formaldehyde and stained with compound **1G**. Images were taken under fluorescence microscope with FITC channel.

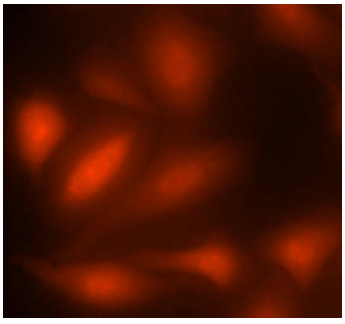


Figure 4. Fluorescence Images of U2OS cells in 96-well Costar black plate were fixed with formaldehyde and stained with compound **1G**. Images were taken under fluorescence microscope with TRITC channel.

between the fluorophore and the reactive group. We find that the spacer of 6-aminohexanoic acid or 4-aminobutanoic acid significantly reduces the fluorescence quenching effect of chlorinated fluorescein on proteins, even at relatively high degrees of labeling. In addition, these novel fluorescent proteins-labeling probes can be conjugated to proteins in the physiological pH range at room temperature and with greater reproducibility. The pH sensitivity of these chlorinated fluorescein dyes in the weakly acidic range (pH 4–6) also makes these dyes useful as pH indicator for acidic organelles of live cells. In order to determine the effectiveness of the probe dyes we labeled U2OS cells with **1G** (Figs. 3 and 4). From these photographs, we can find that it has strong fluorescence and biocompatibility. So, compound **1G** is useful substitute for fluorescein for fluorescence imaging applications. The biological application of compound **2G** is being studied.

Acknowledgments

The project was supported by the National Natural Science Foundation of China (No. 20872117), Specialized Research Fund for the Doctoral Program of Higher Education (No. 200506097003), and the Science and Technology Program Foundation of Xi'an City (No. YF0707).

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7. *The spectral of* **B**: ^{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). Anal. Calcd for $C_{37}H_{37}Cl_6NO_9$: C, 52.13; H, 4.38; N, 1.64. Found: C, 52.08; H, 4.39; N, 1.66. MALDI-TOF MS, *m/z*: 852.52, (calcd: 852.41), IR (cm^{-1}): 2979, 2874, 1775, 1642, 1425, 1214, 1075, 682.*Compound* **C**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.87 (s, 2H, 1′ and 8′-ArH), 8.20 (s, 1H, 5-ArH), 1.44 (s, 18H, *t*-BuH). Anal. Calcd for $C_{31}H_{22}Cl_6O_9$: C, 49.56; H, 2.95. Found: C, 49.52; H, 2.94. MALDI-TOF MS, *m/z*: 751.30, (calcd: 751.22). IR (cm^{-1}): 2977, 1778, 1589, 1454, 1425, 1369, 1212, 1078.
8. *The spectral of* **D**: ^{1}H NMR ($CDCl_3$, 400 MHz) 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). Anal. Calcd for $C_{35}H_{25}Cl_6NO_{11}$: C, 49.56; H, 2.97; N, 1.65. Found: C, 49.61; H, 2.96; N, 1.67. MALDI-TOF MS, *m/z*: 848.25, 751.86, (calcd: 848.29). IR (cm^{-1}): 3365, 2947, 1780, 1739, 1647, 1436, 1373, 1210, 1065, 647.
Compound **1E**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.89 (s, 2H, 1′ and 8′-ArH), 7.86 (s, 1H, 5-ArH), 3.52 (t, 2H, $-CH_2-$), 1.67–1.72 (m, 2H, $-CH_2-$), 1.47–1.51 (m, $-CH_2-$), 2.39 (t, 2H, $-CH_2-$), 1.44 (s, 18H, *t*-BuH). Anal. Calcd for $C_{37}H_{33}Cl_6NO_{10}$: C, 51.41; H, 3.85; N, 1.62. Found: C, 51.39; H, 3.84; N, 1.59. MALDI-TOF MS, *m/z*: 861.11, (calcd: 861.02). IR (cm^{-1}): 3378, 2975, 1778, 1657, 1551, 1454, 1424, 1372, 1211, 1079, 1026, 754.
Compound **2E**: ^{1}H NMR ($CDCl_3$, 400 MHz) 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, *t*-BuH). 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. MALDI-TOF MS, *m/z*: 837.26, (calcd: 836.32). IR (cm^{-1}): 3371, 2977, 1778, 1657, 1547, 1454, 1424, 1373, 1211, 1079, 1026, 754.
Compound **1F**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.87 (s, 2H, 1′ and 8′-ArH), 7.82 (s, 1H, 5-ArH), 3.41 (t, 2H, $-CH_2-$), 1.85∼1.86 (m, 2H, $-CH_2-$), 1.63–1.70 (m, 2H, $-CH_2-$), 2.34 (t, 2H, $-CH_2-$). Anal. Calcd for $C_{27}H_{17}Cl_6NO_8$: C, 46.58; H, 2.46; N, 2.01. Found: C, 46.61; H, 2.44; N, 2.03. MALDI-TOF MS, *m/z*: 696.44, (calcd: 696.14). IR (cm^{-1}): 3395, 2958, 1782, 1658, 1548, 1430, 1216, 1090, 746.
Compound **2F**: ^{1}H NMR ($CDCl_3$, 400 MHz) 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, $-CH_2-$). 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. MALDI-TOF MS, *m/z*: 669.00, (calcd: 668.09). IR (cm^{-1}): 3579, 3333, 1772, 1646, 1559, 1436, 1198, 1150, 1092, 749.
Compound **1G**: From Figure 1 λ_{em} = 550 nm, λ_{ex} = 535 nm, ^{1}H NMR ($CDCl_3$, 400 MHz) δ 11.20 (s, 1H, –COOH), 8.66 (s, 1H, ArOH), 7.91 (s, 1H, 5-ArH), 7.28 (s, 2H, 1′ and 8′-ArH), 3.40 (t, 2H, $-CH_2-$), 1.63–1.71 (m, 2H, $-CH_2-$), 1.52–1.58 (m, 2H, $-CH_2-$), 1.44–1.48 (m, 2H, $-CH_2-$), 2.69 (t, 2H, $-CH_2-$), 2.80 (t, 4H, $-CH_2CH_2-$). Anal. Calcd for $C_{31}H_{20}Cl_6N_2O_{10}$: C, 46.94; H, 2.54; N, 3.53. Found: C, 46.89; H, 2.55; N, 3.49. MALDI-TOF MS, *m/z*: 792.84, (calcd: 793.22). IR (cm^{-1}): 3365, 2947, 1780, 1739, 1647, 1600, 1436, 1210, 1065, 647.
Compound **2G**: From Figure 2 λ_{em} = 550 nm, λ_{ex} = 535 nm, ^{1}H NMR ($CDCl_3$, 400 MHz) 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, $-CH_2CH_2-$). Anal. Calcd for $C_{29}H_{16}Cl_6N_2O_{10}$: C, 45.52; H, 2.11; N, 3.66. Found: C, 45.53; H, 2.10; N, 3.67. MALDI-TOF MS, *m/z*: 766.61, (calcd: 765.16). IR (cm^{-1}): 3318, 2981, 1785, 1732, 1649, 1600, 1430, 1207, 1064, 649.