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Synthesis of chlorinated fluoresceins for labeling proteins

Min Tian, Xiang-Long Wu, Bin Zhang, Jian-Li Li and Zhen Shi*

Department of Chemistry, Northwest University, Xi'an 710069, China

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Abstract—Two novel chlorinated fluoresceins 4,7,2′,7′-tetrachloro-6-(5-carboxypentyl)fluorescein (**8a**) and 4,7,4′,5′-tetra- chloro-6-(5-carboxypentyl)fluorescein (**8b**) were synthesized as fluorescent probes for labeling proteins. These two fluoresceins contain 6-aminohexanoic acid as spacer linker to minimize the fluorescence quenching of the fluorescein molecules by the proteins to be labeled.
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Reactive fluorescent dyes, specially amine-reactive dyes, are most often used to prepare various bioconjugates for immunochemistry,[1] histochemistry,[2] fluorescence in situ hybridization (FISH),[3] cell tracing,[4] receptor binding[5] and other biological applications since amino groups are either abundant or easily introduced into biomolecules.

Selective substitution of xanthene molecules (such as fluoresceins) by chlorine has been proven to be an effective method for developing a fluorescent probe that has narrower emission spectra.[6] The fluorescent probes of narrower emission are a valuable tool for multi-color imaging application such as the high content analysis of live cells.

Compared to the fluorescein molecules that do not contain the linker of 6-aminohexanoic acid, the fluorescence of protein conjugates prepared from compounds **8a** and **8b** are not appreciably quenched, even at relatively high degrees of labeling. The compounds **8a** and **8b** are more photostable than the non-chlorinated fluoresceins.[6] In addition, the lower pK_a of chlorinated fluoresceins make their fluorescence essentially pH insensitive in the physiological pH range.[7,8] These characteristics make the novel chlorinated fluoresceins valuable tools for a variety of biological applications.

The synthesis of compounds **8a** and **8b** were summarized in Scheme 1. Specifically chlororesorcinol and 3,6-dichloro-trimallitic anhydride were condensed with methanesulfonic acid by the molar ratio of 2:1 to get the mixture of 5- and 6-isomer carboxy-functionalized fluoresceins with excellent yields in Scheme 1. Sulfuric acid can also be used for the condensation, but methane sulfonic acid gave a better yield. Next the reaction mixture was heated in pivalic anhydride to give a crude mixture of chlorinated fluorescein dipivalates (compounds **3a**, **3b** or **3c**, **3d**). Compounds **4a**, **4b** (6-iosmers) can be further purified, respectively, by recrystalization in the form of diisopropylamine salts using anhydrous alcohol as the solvent. Through acidification compounds **5a**, **5b** were obtained, and structures and purities of **5a**, **5b** were confirmed by ^{1}H NMR, MOLDI-TOF MS and IR.[9] Compounds **5a** and **5b** were converted to the corresponding succinimidyl esters that were reacted with 6-aminohexanoic acid to give compounds **7a** and **7b**[10] that were first treated with ammonia and followed with HCl neutralization to give compounds **8a** and **8b**.[11] From the emission and absorption maxima of **8a** and **8b** (Figs. 1 and 2) the large Stoke shifts (>15 nm) were shown.

Compounds **8a** and **8b** were converted to their succinimidyl esters that are used for our biological studies since succinimidyl esters are proven to be the best reagents for amine modifications. The amide bonds that formed between compounds **8a** and **8b** and proteins are essentially identical to, and as stable as peptide bonds. These two novel chlorinated fluorescein succinimidyl esters are quite stable if they are properly stored and demonstrate good reactivity and selectivity with aliphatic amines. They had very low reactivity with aromatic amines, alcohols, phenols and histidine.[12]

Keywords: Synthesis; Spacer linker; Labeling proteins.
* Corresponding author. Tel.: +86 13892848785/86 029 88303606; fax: +86 029 88303606; e-mail: tiannminn@163.com

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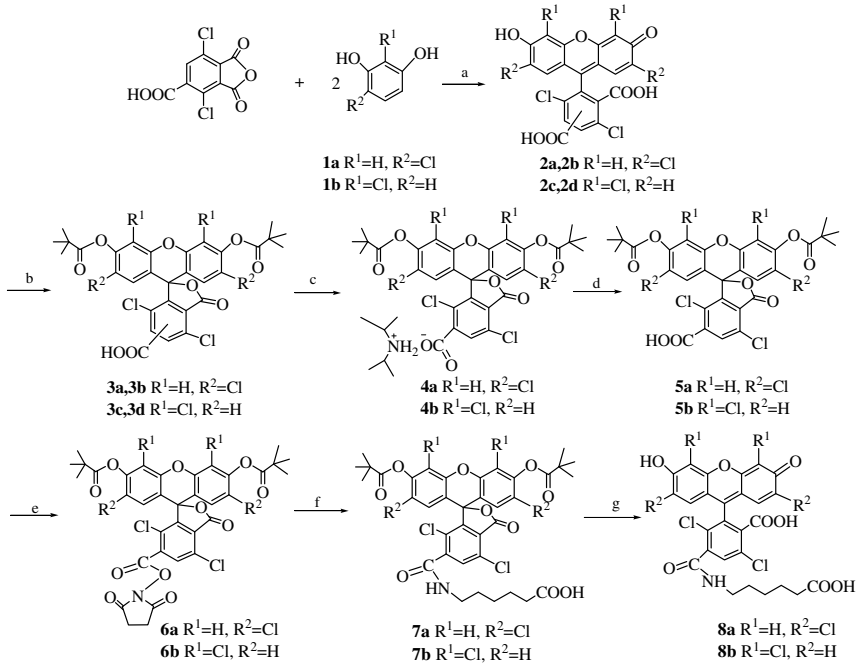

Scheme 1. Synthesis of two new chlorinated fluoresceins **8a** and **8b**. Reagents and conditions: (a) methyl sulfinic acid, N_2, 24 h, 150 °C; (b) pivalic anhydride, reflux; (c) diisopropylamine, anhyd alcohol; **4a**: 39.6%, **4b**: 37.5%; (d) 1 M HCl; **5a**: 90.2%, **6a**: 88.7%; (e) NHS-TFA, Pyridine, CH_2Cl_2 rt; **6a**: 81.5%, **6b**: 84.7%; (f) 6-amino-hexanoic acid, CH_2Cl_2 rt; **7a**: 48.2%, **7b**: 48.1%; (g) i—NH_3/H_2O; ii—1 M HCl; **8a**: 85.3%, **8b**: 87.7%.

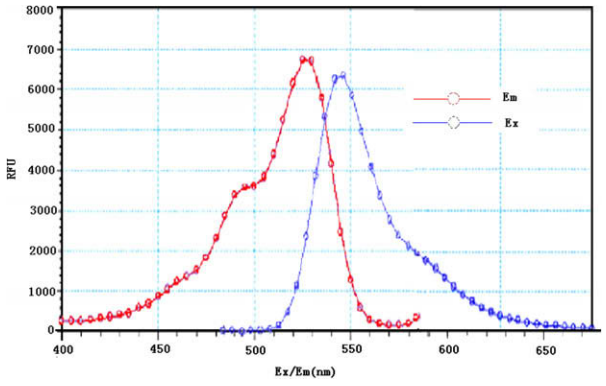

Figure 1. Em/Ex Spectrum of **8a** in 0.1 N NaOH.

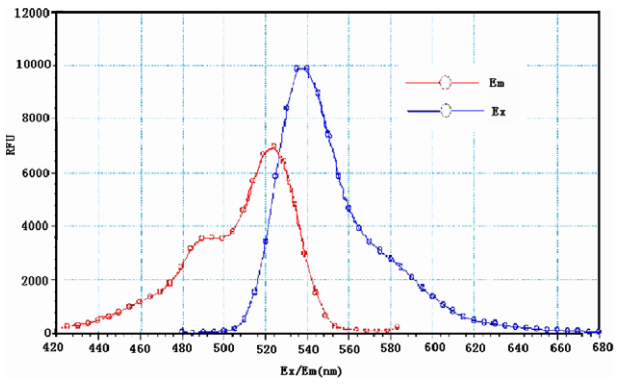

Figure 2. Em/Ex Spectrum of **8b** in 0.1 N NaOH.

Initially we used chlorinated fluoresceins without a linker between the labeling moiety and fluorophore. We observed that the fluorescence of fluoresceins are significantly quenched by the proteins to be labeled. This might result from the electron transfer of electron-rich amino acid residues (such as histidine and tryptophan) to chlorinated fluoresceins. It was reported[13] that to reduce potential interactions between dyes and the phar-

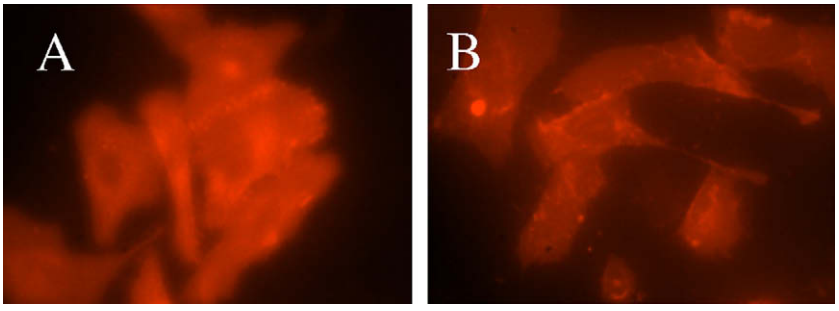

Figure 3. (A,B) Fluorescence Images of U2OS cells in 96-well Costar black plate were fixed with formaldehyde and stained with the succinimidyl esters of compounds **8a** and **8b**. Images were taken under fluorescence microscope with TRITC channel.

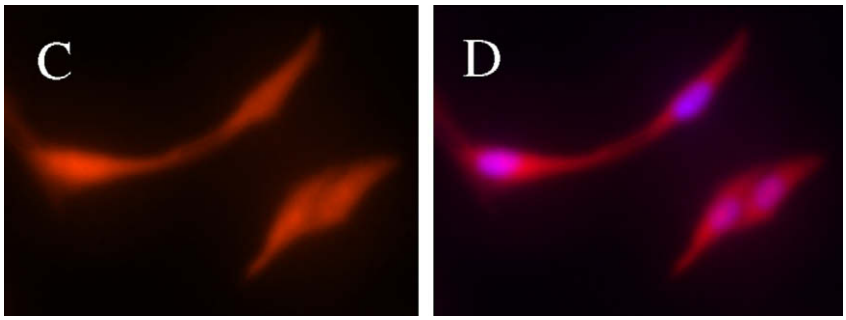

Figure 4. (C,D) Hela cells were fixed with 100 μl of 4% formaldehyde-fixing solution containing 0.5 μM each of the succinimidyl esters of **8a** and **8b** and 1 μM Hoechest 33258 tiat room temperature for 30 min. The cells were washed twice with PBS, and 100 μl PBS was added to fixed cells. Images were taken under fluorescent microscope with TRITC and DAPI channels C (TRITC channel), D (overlay of TRITC and DAPI channel).

macophore of melanocortin receptor, 6-aminohexanioc acid linker was used as optimal length. We find that the linker of 6-aminohexanoic acid significantly reduces the fluorescence quenching effect of chlorinated fluoresceins on proteins. In addition the 6-aminohexanoic acid linker also reduces the interference of antibody bind-site by the fluorescent tags. To determine the effectiveness of the probe dyes we labeled U2OS cells and Hela cells (Figs. 3 and 4). From the photographs, we can find that they have strong fluorescence and biocompatibility.

Acknowledgments

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9. The spectral of **5a**: MALDI-TOF MS, *m*/*z*: 682.89 (calcd 682.33); FT-IR (KBr), υ 2976, 1765, 1478, 1408, 1093 cm^{-1}; ^1H NMR (400 MHz $CDCl_3$) δ 1.40 (s, 18H), 6.91 (s, 2H), 7.13 (s, 2H), 8.18 (s, 1H).
Compound **5b**: MALDI-TOF MS, *m*/*z*: 682.89 (calcd 682.33); FT-IR (KBr), υ 2976, 1765, 1478, 1425, 1092 cm^{-1}; ^1H NMR (400 MHz $CDCl_3$) δ 1.42 (s, 18H), 6.81(d, 2H,*J* = 9.2 Hz), 6.94 (d, 2H,*J* = 9.2 Hz), 8.13 (s, 1H).
10. The spectral of **7a**: MALDI-TOF MS, *m*/*z*: 795.39 (calcd 795.49); FT-IR (KBr), υ 3384, 2974, 1768, 1477, 1408, 1093 cm^{-1}; ^1H NMR (400 MHz DMSO) δ 1.25 (m, 2H), 1.39 (s, 18H), 1.59 (m, 4H), 2.31 (m, 2H), 3.42 (m, 2H), 6.22 (b, 1H), 6.93 (s, 2H), 7.11 (s, 2H), 7.81 (s, 1H).
Compound **7b**: MAL DI-TOF MS, *m*/*z*: 795.60 (calcd 795.49); FT-IR (KBr), υ 3387, 2973, 1784, 1477, 1424, 1092 cm^{-1}; ^1H NMR (400 MHz DMSO) δ 1.27 (m, 2H), 1.41 (s, 18H), 1.69 (m, 4H), 2.31 (m, 2H), 3.41 (m, 2H), 6.20 (b, 1H), 6.82 (s, 2H,*J* = 8.8 Hz), 6.93 (d, 2H, *J* = 8.8 Hz), 7.82 (s, 1H).
11. The spectral of **8a**: from Figure 1 λ_{em} = 525 nm, λ_{ex} = 545 nm, MALDI-TOF MS, *m*/*z*: 628.46 (calcd 627.25); FT-IR (KBr), υ 3352, 2937, 1769, 1632, 1435, 1228 cm^{-1}; ^1H NMR (400 MHz DMSO) δ 1.42 (m, 2H), 1.51 (m, 4 H), 2.19 (m, 2H), 3.17 (m, 2H), 6.93 (s, 2H), 7.17 (s, 2H), 11.99 (s, 1H), 7.82 (s, 1H), 8.70 (s, 1H), 11.20 (s, 2H);^{13}C NMR (400 MHz DMSO): 24.1, 25.9, 27.4, 28.4, 38.9, 79.6, 99.0, 103.5, 107.8, 116.4, 124.4, 128.1, 130.8, 131.8, 144.4, 149.8, 155.4, 163.5, 164.2, 173.3, 174.5.
Compound **8b**: From Figure 2 λ_{em} = 524 nm, λ_{ex} = 540 nm MALDI-TOF MS, *m*/*z*: 628.46 (calcd 627.25); FT-IR (KBr), υ 3352, 2937, 1769, 1632, 1435, 1228 cm^{-1}; ^1HNMR (400 MHz DMSO) δ 1.42 (m, 2H), 1.51 (m, 4H), 2.24 (m, 2H), 3.27 (m, 2H), 6.86 (s, 2H), 7.28 (s, 2H), 7.90 (s, 1H), 8.70 (s, 1H), 11.13 (s, 2H), 12.02 (s, 1H); ^{13}C NMR (400 MHz DMSO): 24.1, 25.9, 27.4, 28.5, 33.6, 80.5, 107.3, 112.9, 124.4, 126.1, 127.7, 130.4, 132.0, 135.7, 141.7, 144.8, 147.5, 149.6, 156.0, 163.7, 174.0.
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