One Pot Synthesis of Isometrically Pure 5-Carboxy-sulforhodamines and Their Application for Labeling Proteins

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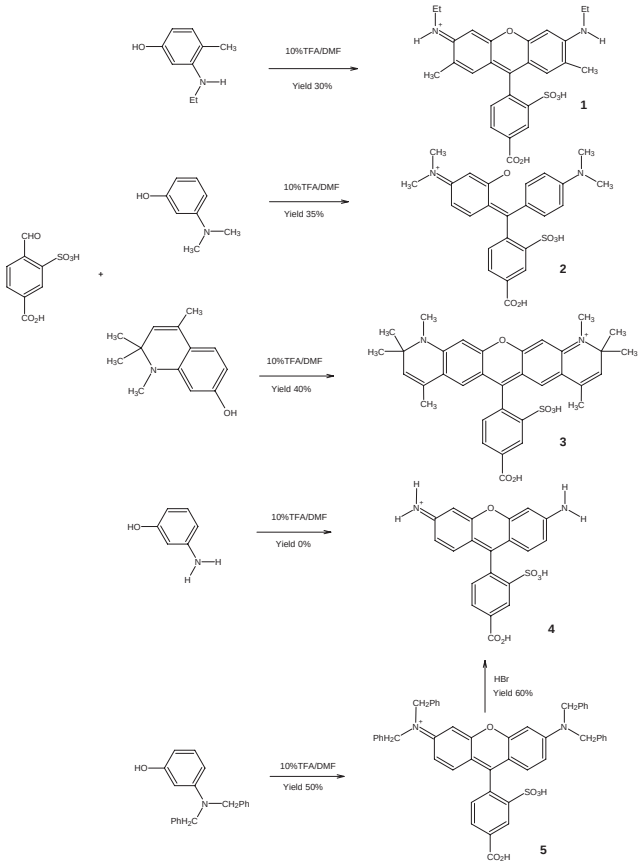
New reactive fluorescent dyes, 5-carboxy-sulforhodamines, were synthesized by one pot synthesis and their labeling proteins were examined.

The utility of fluorescent dyes spans many scientific disciplines from biology to material sciences.[1,2] Of these fluorescent probes, the reactive fluorescein and rhodamine dyes, are widely used in various biological applications. Typically the condensation of 1,2,4-benzenetricarboxylic anhydride with a substituted resorcinol or 3-aminophenol are used to prepare fluorescein or rhodamine dyes.[3,4] However, this method always leads to two regioisomeric products (5- or 6-isomer) that are separated by a tedious chromatographic process. Regioisomerically pure fluorescent probes are preferred in critical biological applications such as labeling biological molecules, DNA sequencing, locating proteins in living cells, detecting a specific functional group of proteins, and measuring intracellular ion concentrations.[1,5] Regioisomerically pure fluorescent dyes give better reproducibility than the corresponding mixed isomers. More recently, Jiao et al. reported the microwave-assisted synthesis of regioisomerically pure bromorhodamine compounds in strong acid condition (H_2SO_4) by two-step reactions.[6]

We envision that a new synthetic method can combine the condensation and oxidation in one pot. The mild acidic reaction condition and homogenous solution are necessary to prevent the decomposition of the products. Here, we extend to report that the usage of TFA in DMF in a precedent method as reaction media can achieve this target.[4] The condensation reactions and oxidation reaction occur in mild acidic condition (10% TFA in DMF) at 80 °C for 12 h as shown in Scheme 1. The oxidation reaction happens by the oxygen in the air without any addition of oxidizers. The moderate yields (from 30 to 50%) were got in this one pot synthesis.[7] We observe that no other fluorescent compounds are formed in this one pot synthesis. Unfortunately, the synthesis of 5-carboxy-sulforhodamine 110 **4** fails by the reaction of 4-carboxy-2-sulfobenzaldehyde with 3-aminophenol. An alternative route to synthesize the compound (**4**) is successful through the reaction of HBr solution with **5**, that compound (**5**) is made by the reaction of 4-carboxy-2-sulfobenzaldehyde with 3-(*N*,*N*-dibenzylamino)phenol in good yield (50%).

All the fluorescent dyes (**1**–**5**) were simply purified by flash silica chromatography using mixed MeOH/$CHCl_3$ solvents. The new fluorescent dyes (**1**–**5**) have intense color in aqueous solution. Their emission spectra are shown in Figure 1. Their maximum absorption and emission wavelengths are similar to those of the commercial dyes such as sulforhodamine 6G, TAMRA/Lissamine rhodamine B and Texas Red. Additionally they are very soluble in aqueous solution. These characteristics make these regioisomerically pure fluorescent dyes ideal fluorescent probes for multi-color detection. The relative quantum yield of these new dyes (**1**–**4**) are essentially identical with rhodamine 110, sulforhodamine 6G, TAMRA, and Texas Red, respectively.



Scheme 1. Synthesis of 5-carboxy-sulforhdamines.

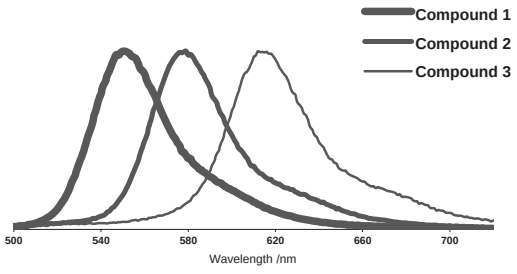


Figure 1. Emission spectra of 5-carboxy-sulforhdamines (compound **1**–**3**) in PBS buffer solution.

We have also used these new dyes to label biological mole-

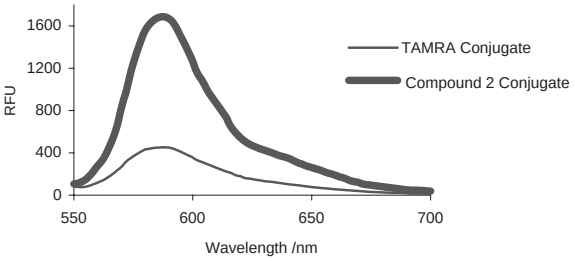


Figure 2. Fluorescence spectra of compound **2**-streptavidin and TAMRA-streptavidin conjugate in PBS buffer solution.

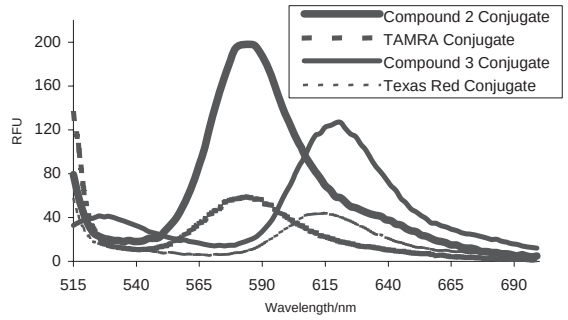


Figure 3. Fluorescence resonance energy transfer (FRET) of streptavidin conjugates with fluorescein-biotin in PBS buffer solution.

cules such as avidins. The fluorescent streptavidin conjugates with these new dyes were readily prepared via their succinimidyl esters. In slightly basic solution (pH = 8.5), the reactions of succinimidyl ester of these dyes with streptavidin give the desired fluorescent conjugates.[8] The dye/protein ratio for these new fluorescent streptavidin conjugates is adjusted to ca. 3 by controlling the ratio of reactive dyes with streptavidin and reaction time as described in literature. The dye/protein ratio is determined according to the literature method.[9] The dye/protein ratio is controlled in the range of from 3 to 4 due to the best performance of fluorescent brightness. Comparison of the compound **2**-streptavidin conjugate (10 nM; D/P = 3.2) with known TAMRA-streptavidin (10 nM, D/P = 3.2) in fluorescent emission (excited at 545 nm) is shown in Figure 2. Compound **2**-strepavidin conjugate demonstrates much higher fluorescence intensity (4 times higher) than TAMRA-streptavidin. That is probably due to the less quenching by the protein to this dye (compound **2**). These sulforhodamine dyes (**1**–**4**) are superior to known rhodamine dyes for the labeling biological molecules. One of the reasons may be due to more negative charge for sulfonic group than carboxyl group.

The high affinity and specificity of avidin–biotin interaction has been exploited for diverse applications. The strong binding of biotin to streptavidin has become a standard tool in molecular biology ($K_D = 10^{-15}$ M).[10] The strong binding of streptavidin conjugates of **2**, TAMRA, **3** and Texas Red (10 nM) with biotin conjugates of fluorescein (40 nM) in PBS buffer solution has also been examined through fluorescence resonance energy transfer (FRET). The FRET spectra is based on excited at 485 nm (fluorescein) as shown in Figure 3. The higher fluorescent intensities of compound **2**-streptavidin and compound **3**-streptavidin conjugates than their analogous commercial conjugates further warrant that these new fluorescent labeling dyes have superior properties. These **2** and **3** streptavidin conjugates are excellent replacement for commercial streptavidin conjugates by TAMRA and Texas Red, two prominent fluorescent labeling dyes.

In summary, the reactive 5-carboxy-sulforhodamines were prepared by one pot synthesis under mild acidic condition. The new reactive fluorescent dyes show superior labeling properties to the known fluorescent rhodamine dyes. The 5-carboxy-sulforhodamines can be widely used as regioisomerically fluorescent labeling reagents for the development of biological or cell-based assay.

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References and Notes

1 R. P. Haughland, "Handbook of Fluorescent Probes and Research Product," 9th ed. (2002).
2 S. W. Zhang and T. M. Swager, *J. Am. Chem. Soc.*, **125**, 3420 (2003).
3 W. C. Sun, K. R. Gee, D. H. Klaubert, and R. P. Haugland, *J. Org. Chem.*, **62**, 6469 (1997).
4 J. Liu, Z. Diwu, W. Y. Leung, Y. Lu, B. Patch, and R. P. Haugland, *Tetrahedron Lett.*, **44**, 4355 (2003).
5 A. Minta, J. P. Kao, and R. Y. Tsien, *J. Biol. Chem.*, **264**, 8171 (1989).
6 G. S. Jiao, J. C. Castro, T. H. Thoresen, and K. Burgess, *Org. Lett.*, **5**, 3675 (2003).
7 4-Carboxy-2-sulfobenzaldehyde was prepared by multiple-step synthesis from 3-bromo-4-methylbenzoic acid. General experimental procedure: 4-Carboxy-2-sulfobenzaldehyde (250 mg; 1 mmol) and 3-aminophenols (2.4 mmol) was dissolved in 10 mL of DMF and 1 mL of TFA. The reaction mixture was stirred and heated up to 80 °C for 12 h in the air environment. The reaction mixture was cooled down and poured into 200 mL of diethyl ether. The crude colorful precipitates were collected and loaded into silica column. The pure products were obtained by eluting with solvents $CHCl_3$:MeOH = 10:1 to $CHCl_3$:MeOH = 3:1. All new compounds (**1**–**5**) were characterized by both ^{1}H NMR and MS. The purity of these products has been determined by analytic HPLC.
8 G. T. Hermanson, "Bioconjugate Techniques," Academic Press, San Diego (1999).
9 C. Lefevre, H. C. Kang, R. P. Haugland, S. A. Malekzadeh, and R. P. Haugland, *Bioconjugate Chem.*, **7**, 482 (1996).
10 V. Buschmann, K. D. Weston, and M. Sauer, *Bioconjugate Chem.*, **14**, 195 (2003).

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