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4-Sulfotetrafluorophenyl (STP) Esters: New Water-Soluble Amine-Reactive Reagents for Labeling Biomolecules

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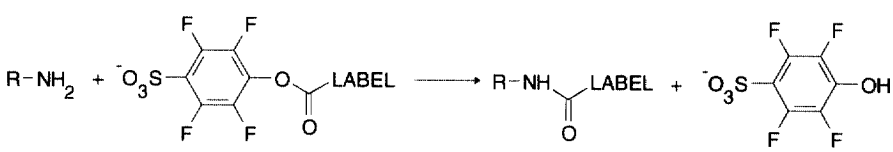
Abstract: 4-Sulfo-2,3,5,6-tetrafluorophenyl (STP) esters of biopolymer-labeling molecules are shown to be dramatically more water-soluble than the corresponding N-hydroxysuccinimidyl (NHS) esters. These STP esters are easily synthesized, and react readily with primary amines to give the same acylation products as NHS and pentafluorophenyl (PFP) esters.

Amine-reactive chemical moieties such as pentafluorophenyl (PFP) and N-hydroxysuccinimidyl (NHS) esters of carboxylic acids have found ubiquitous usage in the field of bioconjugate chemistry. PFP and NHS esters of various labeling molecules such as fluorophores, haptens, photoaffinity labels, crosslinkers, etc., have been attached to biopolymers such as proteins and nucleic acids by acylation of biopolymer amine groups.[1] The resulting amide link between biopolymer and label is chemically stable, allowing for long term use of the labeled compound. One obstacle to this approach is that the labeling molecules often are hydrophobic, and conversion into PFP or NHS esters for attachment to biopolymers only increases this hydrophobicity. Thus, it is difficult to attain high concentrations of the reactive form of the labeling molecules in the aqueous environment of proteins and nucleotides. Organic co-solvents such as DMF or DMSO are sometimes used to improve solubility of the reactive ester, but this may not be optimal for the biopolymer.

The preparation of sulfo-NHS esters was an attempt at making these amine-reactive compounds more water soluble,[2] but they are often difficult to synthesize, and are still more difficult to obtain in highly pure form. A 2-nitro-4-sulfophenol ester was also reported as a water-soluble active ester.[3] It occurred to us to combine the high amine reactivity of perfluorophenyl esters with the water-solubilizing character of sulfonic acid groups in an effort to achieve a novel and useful labeling moiety.



2,3,5,6-Tetrafluorophenol (Aldrich) is readily sulfonated at ambient temperature in fuming sulfuric acid. The sulfonated product is precipitated from water with solid NaCl, then recrystallized from acetonitrile to give analytically pure sodium sulfonate **1** as its dihydrate.[4] The pK_a of the phenolic hydroxyl group in **1** was determined to be 4.5.[5]

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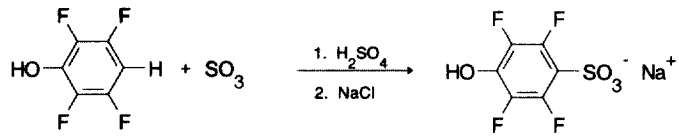

1

Carbodiimide-mediated coupling of **1** with various carboxylic acids was generally successful.[6] Alternatively, ester formation was obtained by first converting **1** into its trifluoroacetate (**2**), followed by reaction with the appropriate carboxylic acid in dry acetonitrile containing pyridine.[7] Flash chromatography on silica gel using $\leq$5% water in acetonitrile, or chloroform/acetone, gave pure 4-sulfo-2,3,5,6-tetrafluorophenyl (STP) esters in 37–92% yield (Table 1).

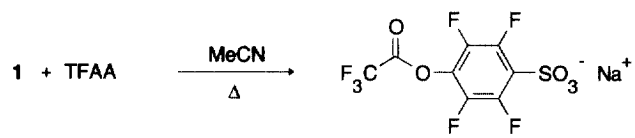

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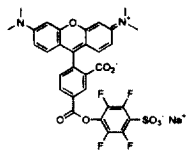
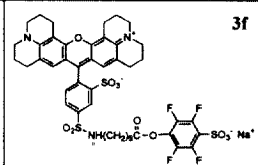
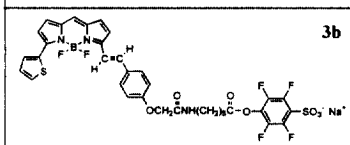
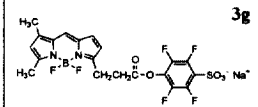
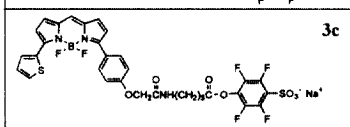
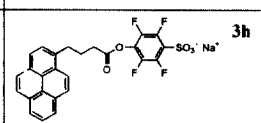
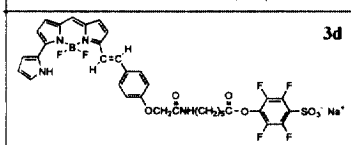
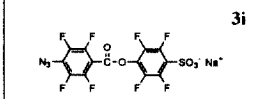
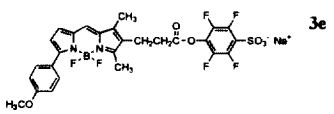
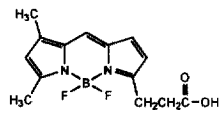
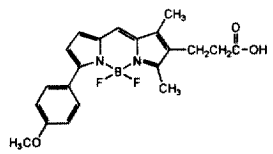
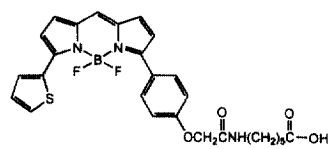
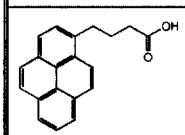
Table 1. Preparations of STP Esters

Compound	Method	% Yield	Compound	Method	% Yield
3a 	carbodiimide	60	**3f** 	TFA	37
3b 	carbodiimide	67	**3g** 	TFA	92
3c 	carbodiimide	71	**3h** 	TFA	47
3d 	carbodiimide	57	**3i** 	TFA	68
3e 	carbodiimide	65			

For highly hydrophobic labels, such as the BODIPY[8] (**3b-e, g**) and pyrene (**3h**) fluorophores, STP esters were 3 orders of magnitude more soluble in water than the corresponding NHS esters (Table 2).

Table 2. Molarity of Saturated Dye-Ester Solutions in Water[a]

Dye	STP ester	NHS ester
	>0.106 (**3g**)	2.2×10^{-4}
	0.0465 (**3e**)	5.86×10^{-6}
	0.0477 (**3c**)	2.65×10^{-5}
	0.0233 (**3h**)	$<2.86 \times 10^{-6}$

[a] Concentrations determined by spectroscopic measurement of absorption at λ_{max} for the dye. Identical extinction coefficient assumed for STP and NHS esters of the same dye.

In a competition experiment, the STP ester **3g** proved more reactive with a primary amine than the corresponding NHS ester. When a limited quantity of benzylamine was added to an equimolar solution of the STP and NHS esters of 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid, the STP ester (**3g**) was preferentially consumed, as judged by TLC analysis.[9]

In conclusion, these STP esters of reactive dyes are significantly more water soluble than the corresponding NHS esters. That, combined with their high amine reactivity, makes them a useful tool for labeling biopolymers.

The authors would like to acknowledge Leslie Bayer for her editorial contributions to this paper.

References & Notes

1. Hermanson, G. T. *Bioconjugate Techniques*; Academic Press: New York, 1996.
2. Staros, J.V., *Biochemistry* **1982**, *21*, 3950.
3. Aldwin, L. and Nitecki, D., *Anal. Biochem.* **1987**, *164*, 494.
4. 2,3,5,6-Tetrafluorophenol (10.1 g, 61.4 mmol) was taken up in 22 mL fuming sulfuric acid (30% SO_3) and stirred at ambient temperature for 18 hours before pouring the mixture into 200 mL iced brine. The product was precipitated by stirring in solid NaCl until no further precipitate formed. This mixture was filtered through a sintered glass disc and the collected solids were taken up in 330 mL boiling CH_3CN,

filtered through paper while hot, and allowed to cool slowly to ambient temperature. The colorless crystalline product was collected by filtration, yielding 9.64 g (64%) after drying *in vacuo*. Anal. calcd. for $C_6HF_4NaO_4S \cdot 2\ H_2O$: C, 23.69; H, 1.66; Found: C, 23.71; H, 1.97. ^{13}C NMR (D_2O): δ 114.8 (t, J=16.1; C1), 140.8 (dd, J=243, C2,6), 141.0 (t, J=14.1; C4), 146.6 (dt, J=247; C3,5). ^{19}F NMR (CD_3CN) Φ ($CFCl_3$) 140.6 (m, 2F), 159.1 (m, 2F).

5. The acidity constant for the ionization of **1** to its phenoxide form was determined spectroscopically. By monitoring the absorbance (225–300 nm) of buffered solutions of **1** in the range pH 2.0–12.0, λ_{max} of the phenoxide was found to be 243 nm. The protonated form showed λ_{max} <225 nm. Aliquots of a stock solution of STP were then diluted with 50 mM phosphate buffers (pH 2.20, 3.12, 4.18, 5.15, 6.02, 7.00, 7.97, 9.00, 10.00, 11.00, 12.00) and the optical density (O.D.) of each was measured at 243 nm. Plotting the measured O.D. vs. pH produced a titration curve showing an inflection point (pK_a) at pH 4.5.
6. For example, to a solution of 6-(((4-(4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-yl)phenoxy)acetyl)amino)hexanoic acid (U.S. Patent 5,274,113) (250 mg, 0.47 mmol) and 4-sulfotetrafluorophenol, sodium salt (**1**, 135 mg, 0.50 mmol) in 1 mL DMF and 15 mL acetone was added 1,3-dicyclohexylcarbodiimide (115 mg, 0.55 mmol) and the mixture was stirred at room temperature for 20 hours. The resulting precipitate was removed by filtration and the filtrate was concentrated under reduced pressure to give a crude product. The product was purified by column chromatography on silica gel with 40% chloroform in acetone as eluant to give 265 mg (71%) of the STP ester **3c** as a dark purple solid; one spot on TLC (R_f = 0.42, 25 % methanol in chloroform); ^1H NMR (DMSO-d_6): δ 8.20–8.13 (m, 1H, NH), 8.05 (d, 1H, ArH), 8.03 (d, 2H, ArH), 7.84 (d, 1H ArH), 7.74 (s, 1H ArCH=), 7.39 (d, 1H, ArH), 7.35 (d, 1H, ArH), 7.28-7.22 (m, 1H, ArH), 7.09 (d, 2H, ArH), 7.01 (d, 1H, ArH), 6.92 (d, 1H, ArH), 4.59 (s, 2H, OCH_2), 3.20–3.12 (m, 2H, CH_2), 1.40–1.31 (m, 2H, CH_2), 1.72–1.65 (m, 2H, CH_2), 1.54–1.47 (m, 2H, CH_2), 1.40–1.31 (m, 2H, CH_2); absorption maximum, 587 nm (ε 61,100 $cm^{-1}M^{-1}$), emission maximum, 618 nm in acetonitrile. Anal. calcd. for $C_{33}H_{25}BF_6N_3NaO_7S_2 \cdot 2H_2O$: C, 49.11; H, 3.40; N, 5.21. Found: C, 49.11; H, 3.40; N, 4.92.
7. 4-Sulfotetrafluorophenyl trifluoroacetate (**2**) was synthesized by refluxing 4-sulfotetrafluorophenol, sodium salt (**1**, 5.00 g, 16.4 mmol) with 35 mL trifluoroacetic anhydride in 300 mL CH_3CN for 1 hour, while the solution was protected from moisture with a $CaCl_2$ drying tube. Volatiles were distilled off under reduced pressure at 30 °C and dried *in vacuo* to yield 5.48 g **2** as a colorless flaky residue: ^{19}F NMR (CD_3CN) Φ 69.9 (s), 133.8 (m), 149.8 (m). In this example, **2** (1.36 g, 3.73 mmol) and pyridine (0.2 mL, 2.47 mmol) were added to a solution of 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid (U.S. Patent 4,774,339) (500 mg, 1.71 mmol) in 300 mL dry CH_3CN. The resulting mixture was stirred for 5 hours at 35 °C before reducing the volume to 50 mL by concentration under reduced pressure at 35 °C. Precipitated solids were filtered off, and 15 g silica was added to the filtrate. The mixture was concentrated to dryness *in vacuo*, and the residue purified by column chromatography on silica, eluting with 2% H_2O/CH_3CN. The collected fractions, which were pure by TLC (R_f 0.71-1.0 in 5% H_2O/CH_3CN) were pooled and concentrated to dryness under reduced pressure at 30 °C to yield 0.85 g (92%) **3g**, a red–orange solid: ^1H NMR (CD_3CN) δ 2.25 (s, 3H), 2.50 (s, 3H), 3.15 (t, 2H), 3.30 (t, 2H), 6.25 (s, 1H), 6.35 (s, 1H), 7.00 (s, 1H), 7.40 (s, 1H); ^{19}F NMR (CD_3CN) Φ ($CFCl_3$) 136.0 (m), 140.2 (q), 149.9 (m). Anal. calcd. for $C_{20}H_{14}BF_6N_2NaO_5S \cdot 0.5H_2O$: C, 43.58; H, 2.74; N, 5.08. Found: C, 43.62; H, 2.72; N, 5.23.
8. BODIPY is an abbreviation of borondipyrromethene difluoride (4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene) and a registered trademark of Molecular Probes, Inc. a) Kang, H.C., Haugland, R.P., Fisher, P.J., and Prendergast, F.G. *Proc. SPIE-Int. Soc. Opt. Eng.* **1989**. *1063*, 68; b) Karolin, J., Johansson, L. B.-A., Standberg, L. and Ny, T. *J. Am. Chem. Soc.* **1994**, *115*, 7801.
9. To a solution of **3g** (6.7 mg, 0.013 mmol) and the corresponding NHS ester (5.0 mg, 0.013 mmol) in THF (1.0 mL, distilled) at room temperature was added benzylamine (1.4 μL, 0.013 mmol). The resulting solution was stirred and analyzed by TLC (0.5% HOAc/THF) at 2, 15, 30, 45, 55, 65 and 75 minutes. The STP ester **3g** (R_f 0.46) was preferentially consumed over the NHS ester (R_f 0.75); by 45 minutes all the STP ester was gone, while the NHS ester was still present. Only a trace of hydrolysis product was observed. The R_f of the benzylamide product was 0.80.