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Novel Derivatization of Protein Thiols With Fluorinated Fluoresceins

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Abstract: Fluorescein diacetate analogs **3a-3c** are shown to react selectively with protein sulfhydryl groups at neutral pH in aqueous solution, by means of substitution at the perfluorinated ring. The acetate moieties are cleaved by treatment with aqueous carbonate or by endogenous esterases, giving a fluorescent protein conjugate. Copyright © 1996 Elsevier Science Ltd

Traditional derivatization of free sulfhydryl groups in proteins with fluorescent reagents involves reaction with fluorochromes containing haloacetamides, maleimides, aziridines, halomethylaryl groups, and pyridyl disulfides.[1] Of these, fluorescent iodoacetamides are used most frequently because of the speed of reaction at neutral pH.[2] However, iodoacetamides suffer from instability to light, particularly in solution. Also, iodoacetamides can be susceptible to oxidation in DMSO, a widely used co-solvent for the preparation of fluorochrome-protein conjugates. Additionally, iodoacetamides are often difficult to prepare in pure form, sometimes necessitating the use of chloroacetamides as intermediates.[3] Thus, there is still a substantial need for improved fluorescent, sulfhydryl-reactive probes.[4] Herein we report a novel means of derivatizing protein thiol groups involving nucleophilic aromatic substitution of fluorinated fluoresceins.

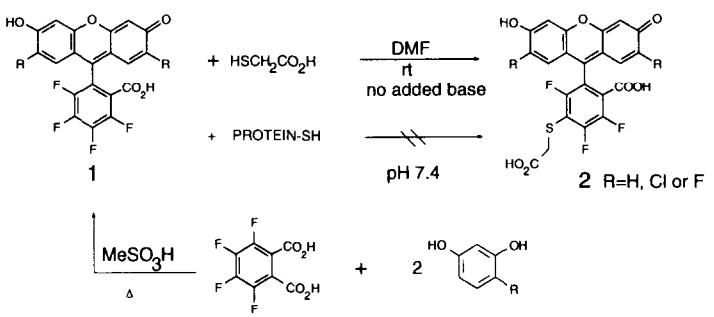

Figure 1

Nucleophilic displacement of activated aromatic fluorines is well known. A critical requirement for facile reaction is the presence of an electron-withdrawing group *ortho* or *para* to the fluorine atoms.[5] Reaction of the fluorinated fluorescein **1**[6] with thiols such as mercaptoacetic acid in organic solvents was facile *in the absence of added base*.[7] The reaction was highly selective for substitution at the 6-position, *para* to the carboxylic acid (Figure 1). However, at near neutral pH in water, no reaction was observed between **1** and *E. coli* β-galactosidase, a thiol-containing enzyme. We rationalized this result by assuming that the carboxylic acid is mostly deprotonated at neutral pH in aqueous solution, thus deactivating the tetrafluorinated ring of **1** toward substitution. In organic solution the carboxylate is protonated and thus electron-withdrawing, allowing for facile *para*-substitution (Figure 1).

Acetylation of the phenolic hydroxyl groups is a way to stabilize the electron-withdrawing character of the carboxylate in aqueous solution, by forcing the dye into a nonfluorescent spirolactone form **3** (Figure

2).[8] Gratifyingly, reaction of β-galactosidase with **3a** at near neutral pH readily resulted in protein modification (Figure 2). Simply raising the pH briefly to 10 cleaved the acetates from the dye, generating the fluorescently labeled protein **5a**.[9] Purification by gel-filtration chromatography readily separated the protein conjugate from free dye, and allowed reaction stoichiometry to be determined.

High selectivity for sulfhydryl groups is evidenced by the lack of reaction between **3a** and IgG (contains only cystine residues), and between **3a** and streptavidin (contains no cysteine residues), under identical conditions. In contrast, pretreatment of IgG with dithiothreitol (DTT) to liberate free sulfhydryl groups gave the expected reaction product (**5a**) with **3a**.

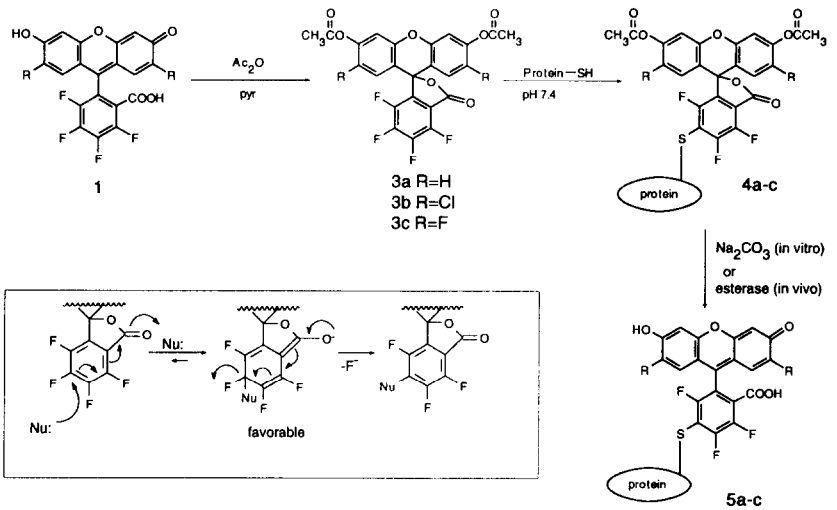

Figure 2

We also prepared the 2′,7′-dichloro (**3b**, R=Cl) and 2′,7′-difluoro (**3c**, R=F) analogs. Table 1 shows the relative reactivity with β-galactosidase of all three probes, as measured by the degree of substitution, from a representative set of experiments.[10] In addition to reaction with proteins *in vitro*, diacetates **3** were also shown to react with biomolecules in intact cells. Cultured human lymphoid B-cells were incubated with 1 μM solutions of **3**, followed by washing with fresh medium.[11] For quantitation of dye binding to cellular macromolecules, the cells were fixed by formaldehyde treatment and analyzed by flow cytometry (Table 1). In this case it is probable that endogenous esterases cleave the acetates from the fluorochrome, obviating the basic hydrolysis step. To confirm that the dyes were labeling cellular proteins, the labeled cells were lysed, and the lysate was analysed by gel electrophoresis,[12] which showed that a number of fluorescently labeled protein bands had been produced (data not shown). Qualitatively, the bands from cells stained with **3a** appeared brighter than those stained with **3b** and 3c, consistent with the fluorescence intensity values determined by flow cytometry and *in vitro* protein labeling results.

In conclusion, we have developed fluorinated fluorescein diacetates (**3**) that readily and selectively react with protein sulfhydryl groups in a novel protein conjugation method. The ease of synthesis and purification of **3** makes these reagents more attractive than traditionally used thiol-reactive fluorescent probes. These reagents add a new means for selective protein derivatization to the repertoire of the bioconjugate chemist.

Table 1. Labelling of Proteins in Vitro and in Cells with 3a-3c

	DOS [a]	DOS [b]	Signal [c]
Dye 3a	5.0	8.6	2450±88
Dye 3b	2.3	4.4	1470±66
Dye 3c	2.8	3.9	400±26
Control (no dye)			2±1

DOS = Degree of substitution.
a 10:1 Dye:protein molar ratio **b** 20:1 Dye:protein molar ratio
c Relative fluorescence emission intensities of cell samples stained in parallel with 1 μM dye, measured by flow cytometry[11]; n=4

References & Notes

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4. For examples of recent efforts, see: a) Langmuir, M.E.; Yang, J.-R.; Moussa, A.M.; Laura, R.; LeCompte, K.A. *Tetrahedron Letters* **1995**, 36, 2989–2992; b) Walker, M.A. *J. Org. Chem.* **1995**, 60, 5352–5355; c) Corrie, J.E.T. *J. Chem. Soc. Perkin Trans. 1* **1994**, 2975–2982.
5. Bolton, R.; Sandall, J.P.B. *J. Chem. Soc. Perkin Trans. II* **1978**, 1288–1292, and references cited therein.
6. For example, tetrafluorophthalic acid (3.72g, 15.6 mmol) and resorcinol (3.43 g, 31.2 mmol) were heated in methanesulfonic acid (50 ml) for 48 hours at 80-90°C. The reaction solution was poured into 400 ml water. After cooling, the precipitate was filtered, rinsed with water (100 ml), and dried *in vacuo* to give 3.68 (91%) of a brick-red powder: ^{1}H NMR (d_6-DMSO) δ 7.00 (d, J=8.7, 2H), 6.70 (d, J=2.3, 2H), 6.60 (dd, J=8.7, 2.3, 2H); ^{19}F NMR (d_6-DMSO) Φ 135.5 (m, 1F), 139.6 (m, 2F), 147.5 (t, 1F); ϵ 85, 600 cm $^{-1}$M^{-1} (508 nm, pH 9). Anal. calcd for $C_{20}H_8O_5F_4$: C, 59.42; H, 1.99. Found: C, 59.39; H, 2.01. Resorcinol and 4-chlororesorcinol are commercially available. For a synthesis of 4-fluororesorcinol, see Patrick, B.T.; Darling, D.L. *J. Org. Chem.* **1986**, 51, 3242-3244.
7. For example, a solution of **1** (R=F, 0.75 g, 1.70 mmol) and mercaptoacetic acid (0.13 mL, 1.80 mmol) in DMF (9 mL) is stirred for 24 hr at rt, then poured into 100 mL cold water. The resulting precipitate is collected, rinsed with water, and dried *in vacuo* to give **2** (R=F) as 0.86 g (99%) of an orange powder: ^{1}H NMR (d_6-DMSO) δ 7.05 (t, J=11.1 Hz, 2H), 6.88 (dd, J=7.5, 2.8 Hz, 2H), 3.84 (s, 2H). ^{19}F NMR (d_6-DMSO) Φ 110.65 (br s, 1F), 120.62 (d, 1H), 134.84 (br s, 2F), 138.15 (br s, 1F). Anal. calcd for $C_{22}H_9F_5O_7S{\cdot}H_2O$: C, 49.82; H, 2.09. Found: C, 49.56; H, 2.32. The reaction proceeded more slowly in acetonitrile.
8. For example, **1** (R=H, 45.1 g, 0.111 mol) is heated briefly with ten equivalents of acetic anhydride in pyridine (100 mL). After cooling, water (300 mL) is added to precipitate **3a** (R=H), which is collected and crystallized from ethyl acetate/hexanes to give the pure product as 43.1 g (80%) of a colorless powder: ^{1}H NMR ($CDCl_3$) δ 7.15 (d, J=22 Hz, 2H), 6.99 (d, J=8.7Hz, 2H), 6.92 (dd, J=8.7, 2.2 Hz, 2H), 2.31 (s, 6H); ^{19}F NMR ($CDCl_3$) Φ 137.26 (m, 1H), 140.79 (m, 1H), 141.21 (m, 1F), 148.98 (m, 1F). Anal. calcd for $C_{24}H_{12}F_4O_7$: C, 59.03;

H, 2.48. Found: C, 59.00; H, 2.69.

9. In a typical coupling experiment, *E. coli* β-galactosidase (Boehringer Mannheim, EC 3.2.1.23, 3 mg) was dissolved in 150 μL 0.1 M phosphate buffered saline (PBS) at pH 7.5. To this was added 13.6 μL of a stock solution of **3a** (5 mg in 500 μL DMSO), giving a molar ratio of 20:1 dye:protein. After 1 hr, TLC showed both **4a** and **3a**, and the pH was raised by addition of 200 μL of 1 M Na_2CO_3 to pH 10. After another 2 hr, the unreacted dye was removed from the conjugate using a P-30 spin column and pH 7.5 PBS. The conjugate contained no free dye, as judged by TLC analysis. The absorbance maxima of the conjugated dye (515 nm) and native protein (280 nm) were measured at pH 7.5. The absorbance at 280 nm was corrected for the absorbance of the free dye (**1**) at 280 nm and the degree of substitution (DOS) calculated using an extinction coefficient of 85,600 $cm^{-1}M^{-1}$ at 515 nm for the free dye (**1a**).
10. *E. coli* β-galactosidase contains 64 cysteine residues/enzyme, (Craven, G.R.; Steers, E.; Anfinsen, C.B. *J. Biol. Chem.* **1965**, 240, 2468–2477) with varying amounts oxidized as cystine bridges, depending on preparation and purification methods. According to the manufacturer, the lots used for the experiments described herein were determined to contain 16.0 free sulfhydryl groups/molecule. Titration with thiol reagents has demonstrated that modification of a large number of cysteinyl residues occurs without loss of enzyme activity (Wallenfels, K.; Müller-Hill, B.; Dabich, D.; Streffer, C.; Weil, R. *Biochem. Z.* **1964**, 340, 41–55).
11. Human B-cells were cultured in RPMI 1640 medium suplemented with 10% PBS, 100 U penicillin, 100 μg streptomycin and 300 μg L-glutamine per mL of culture medium. Stock solutions of **3** in DMSO were added to cell culture medium to obtain a final dye concentration of 1 μM. The cells were incubated at 37 °C for 30 min, centrifuged, then the staining solution was discarded. The pellets were gently resuspended in PBS and incubated for 15 min, then centrifuged again and resuspended in either fresh PBS solution (for cells to be lysed) or in warm 3.7% formaldehyde in PBS for fixation. The cells in formaldehyde were incubated twice for 10 min, centrifuged, and resuspended in fresh culture medium for flow cytometry using a FACS™-Vantage instrument equipped with an argon-ion laser (488 nm excitation); fluorescence emission was collected using a 515 nm longpass filter and a single photomultiplier tube. The sheath fluid was 0.9% NaCl, and typical sample flow rates were between 200 and 400 particles per second.
12. For gel electrophoretic analysis, labeled cells in PBS were heated briefly with SDS gel loading buffer at 90 °C, then allowed to cool and loaded onto 15% (37.5:1) polyacrylamide, 0.05% SDS gels. After electrophoresis, gels were placed directly on a UV transilluminator (300 nm) and photographed. Subsequently, the gels were stained with SYPRO® Orange, a fluorescent protein gel stain, to confirm that the bands labelled with **3** were indeed high molecular weight components and not small molecule thiols such as glutathione (Steinberg, T.H.; Jones, L.J.; Haugland, R.P.; Singer, V.L. *Anal. Biochem.* **1996**, 239, 223-237; Steinberg, T.H.; Haugland, R.P.; Singer, V.L. *Anal. Biochem.* **1996**, 239, 238-245).

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