

Synthesis of a FAsH-Alexa Fluor 568 Fluorescent Probe

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Background

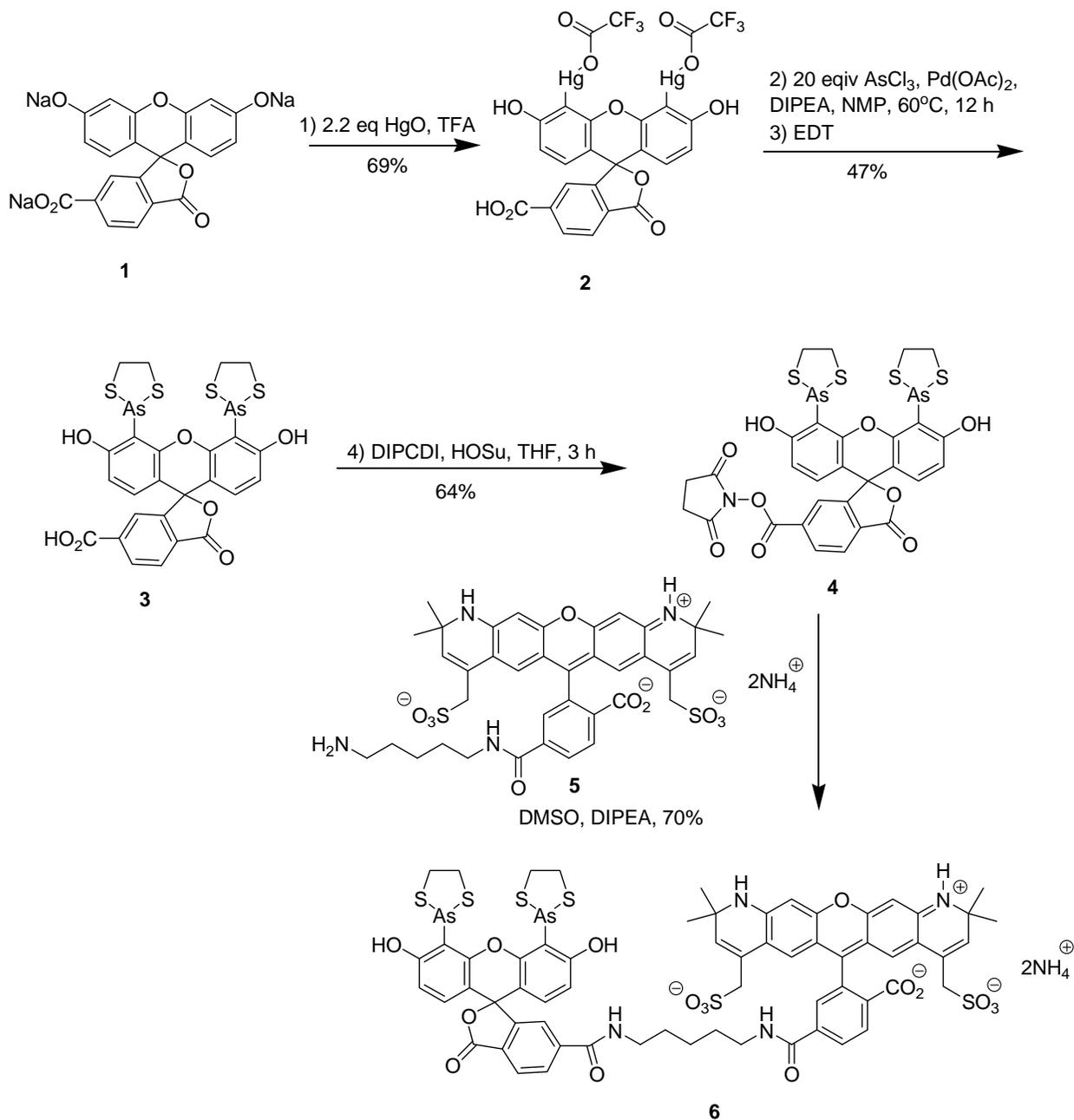
Derivatives of the fluorophore FAsH, used for specific labeling of tetracysteiny- (TC) motifs in proteins, were prepared in order to study modes of prion infection, which are important in a number of human and animal diseases.¹ Critical unresolved questions include understanding mechanisms of uptake, intra- and inter-cellular trafficking of prions, and establishing the sub-cellular compartments where prion replication occurs. With an understanding of how prion infection occurs and is spread between cells, methods may be developed to inhibit these processes and create therapies for these fatal infections. The TC motif specifically binds to biarsenical compounds built on either fluorescein (Lumio Green or FAsH) or resorifin (Lumio Red or ReAsH) dyes, with an enhancement of fluorescence on binding the TC motif. We set out to prepare a conjugate of 6-carboxyflash-EDT₂ succinimidyl ester and an amine-containing Alexa Fluor® compound (Alexa Fluor® 568 cadaverine) to generate a new agent that retains good TC binding specificity and adds the superior fluorescent properties of an Alexa Fluor® dye.

Chemistry

The synthesis of the 6-carboxy FAsH-Alexa Fluor® 568 cadaverine conjugate is outlined in **Scheme 1**. According to original published literature, mercuration of 6-carboxyfluorescein trisodium salt (**1**) with mercuric acetate produced a mono-, di-mercuration mixture (1:3).² This proved troublesome in the next transmetalation step. Without purification of the complex, our yields of the desired arsenical product were <10% and involved tedious chromatography. This methodology was modified³ using an alternate substrate, dimercuric triflate (**2**), which was readily prepared from the exclusive dimercuration of (**1**) using mercuric oxide and trifluoroacetic acid (TFA) in a yield of 69%. Transformation of dimercurate (**2**) to its diarsenical species was performed in the presence of arsenic trichloride and palladium acetate at 60°C. Immediate treatment of (**2**) with excess 1,2-ethanedithiol (EDT) led to a 47% yield of intermediate (**3**).

Using 1,3-di-isopropylcarbodiimide (DIPCDI) and *N*-hydroxysuccinimide (NHS), the free carboxylic acid (**3**) was converted to the activated succinimidyl ester (**4**) with a 64% yield. The activated acid (**4**) was conjugated with 568 Alexa Fluor® 6-carboxyl cadaverine to give the final imaging agent (**6**) in a 70% yield.

Scheme 1. Synthesis of 6-carboxy FIAsH-cadaveryl-Alexa Fluor® 568 conjugate



Experimental

General. Silica gel 60 F254 TLC plates were from Merck. Flash chromatography was done on an Analogix Intelliflash Workstation with a SuperFlash Septra Si 50 SF column. Mass spectral analyses were done on an Agilent 1200 mass spectrometer and $^1\text{H-NMR}$ obtained on a Varian 400 MHz. Chemical shifts are reported in parts per million (δ) and are referenced to tetramethylsilane (TMS). Most starting materials are commercially available from Aldrich, while the Alexa Fluor® dyes are available from Invitrogen Corporation.

6-Carboxyfluorescein-4',5'-bis(mercuric trifluoroacetate) (2). 6-Carboxyfluorescein trisodium salt (**1**) (848 mg, 1.92 mmol) was added to a solution of mercuric oxide (917 mg, 4.22 mmol) in trifluoroacetic acid (10 mL). After stirring for 24 hours, the yellow mixture was evaporated and diluted with water (50 mL). The precipitate was collected by filtration and dried *in vacuo* over phosphorus pentoxide (P_2O_5) to constant weight, to give (**2**) as a red powder with a yield of 1.32 g (69%). Mp: $>300^\circ\text{C}$; $^1\text{H NMR}$ ($\text{D}_2\text{O}+\text{Na}_2\text{CO}_3$, 400 MHz) 8.01 (dd, 1 H, $J = 1.6$ Hz, $J = 8.0$ Hz), 7.79 (d, 1 H, $J = 8.0$ Hz), 7.68 (s, 1H), 7.13 (d, 2H, $J = 9.2$ Hz), 6.64 (d, 2H, $J = 9.2$ Hz).

4',5'-Bis(1,2,3-dithioarsolan-2-yl)-fluorescein-6-carboxylic acid (3). Compound (**2**) (502 mg, 0.50 mmol) was suspended in anhydrous *N*-methyl-2-pyrrolidone (NMP) (6 mL) under an argon atmosphere. To this suspension was added arsenic (III) trichloride (0.85 mL, 10 mmol), palladium (II) acetate (40 mg) and *N,N'*-diisopropylethylamine (DIPEA) (0.70 mL, 4.0 mmol). The orange suspension quickly changed to a clear yellow solution. The resulting solution was stirred at 60°C for 13 hours as it turned into a dark red suspension. This suspension was poured into a mixture of aqueous potassium phosphate buffer (125 mL, 0.2 M, pH = 7) and acetone (125 mL) and treated with ethanedithiol (EDT) (2.5 mL). After stirring for 30 minutes, chloroform (CHCl_3) (125 mL) and acetic acid (AcOH) (6 mL) were added to the heavy brown mixture and the suspension was stirred for an additional 1 hour before separation. The aqueous layer was extracted with CHCl_3 (2 X 125 mL). The combined organic layer was washed with 5% AcOH (100 mL) and dried over anhydrous sodium sulfate (Na_2SO_4). Evaporation of the solvents gave an orange residue which was purified by flash chromatography using a gradient of 0.5% AcOH in toluene to 0.5% AcOH in toluene:ethyl acetate (6:1) to obtain product (**3**) as a pale pink solid

with a yield of 168 mg (47%). [R_f 0.36 in 0.5% AcOH toluene:ethyl acetate (1:1)]. Mp: 184-185 °C. ^1H NMR (d^6 -dimethylsulfoxide (DMSO), 400 MHz) δ 13.60 (br, 1H), 10.64 (s, 2H), 8.22 (dd, 1H, $J = 1.0$ Hz, $J = 8.0$ Hz), 8.20 (d, 1H, $J = 8.0$ Hz), 7.64 (s, 1H), 6.59 (d, 2H, $J = 8.6$ Hz), 6.54 (d, 2H, $J = 8.6$ Hz), 3.35-3.26 (m, 8H); MS (m/z): 708.9 (M-H) $^-$.

4',5'-Bis(1,2,3-dithioarsolan-2-yl)-fluorescein-6-carboxylic acid, succinimidyl ester (4).

Compound 3 (60 mg, 84 μmol) was dissolved in anhydrous tetrahydrofuran (THF) (8 mL) under an argon atmosphere. To the solution was added DIPC DI (34 μl , 100 μmol) and NHS (24 mg, 100 μmol). After 3 hours, the mixture was evaporated and the residue was purified by flash chromatography using a gradient of 0.5% AcOH in toluene:ethyl acetate (10:1 to 4:1) to give product (4) as an orange solid with a yield of 43 mg (64%). R_f 0.53 in 0.5% AcOH in toluene:ethyl acetate (1:1). ^1H NMR (CDCl_3 , 400 MHz) δ 9.98 (s, 2H), 8.38 (d, 1H, $J = 8.0$ Hz), 8.14 (d, 1H, $J = 8.0$ Hz), 7.95 (s, 1H), 6.59 (d, 2H, $J = 8.8$ Hz), 6.55 (d, 2H, $J = 8.6$ Hz), 3.65-3.53 (m, 8H), 2.90 (s, 4H); MS (m/z): 805.9 (M+H) $^+$.

Coupling to Alexa Fluor® 568 6-carboxycadaverine. Compound (4) (4.3 mg, 5.3 μmol) was dissolved in DMSO (250 μL). To the solution was added Alexa Fluor® 568 6-carboxycadaverine (5) (6.0mg, 7.4 μmol) and DIPEA (1.4 μL , 8.0 μmol). The resulting solution was shaken for 24 hours at room temperature and monitored by TLC and HPLC until completion. The solution was purified *via* preparative TLC (developed in $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (2:1) system) to give product (6) as a red solid with a yield of 5.6 mg (70%). R_f 0.47 in $\text{CH}_2\text{Cl}_2:\text{MeOH}$ (1:1). Analysis of this product was performed using an Agilent 1200 Series High Performance Liquid Chromatography System. A = 0.05% TFA in water and B = 0.05% TFA in acetonitrile. An Agilent XDB-C₁₈, 3.5 μm , 3.0 x 150 mm column was used with a flow rate of 1.0 mL/min. A linear gradient from 5-85% acetonitrile was used with the 568 Alexa Fluor® cadaverine starting material eluting at 7.8 minutes and the product 6 eluting at 14.4 minutes, $\lambda = 568$ nm. Silica gel 60 F254 TLC plates were from Merck. Final HPLC traces are illustrated in **Figure 1**. The overall yield was 14.5%.

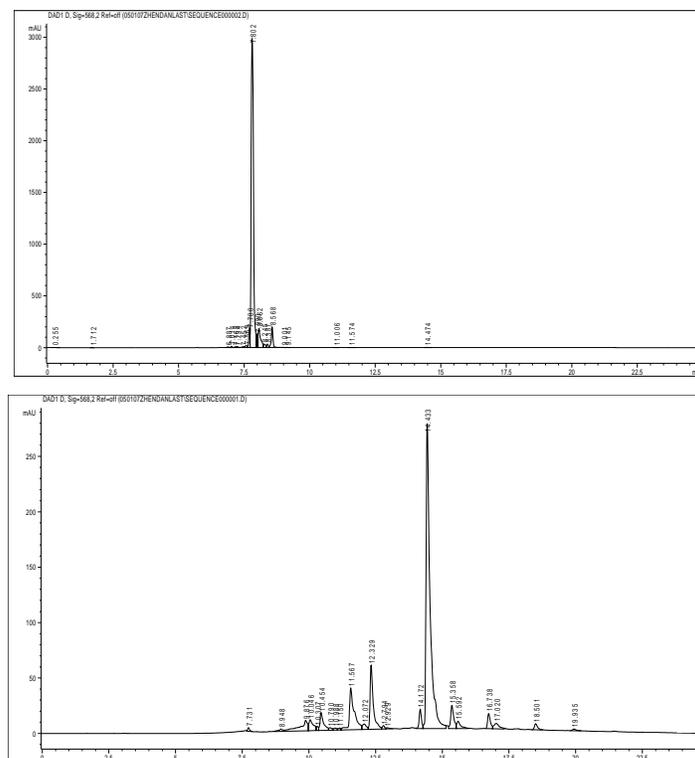


Figure 1. HPLC elution traces of Alexa Fluor® 568 6-carboxycadaverine starting material (top) and the final product, the 6-carboxy FlAsH-568 cadaveryl Alexa Fluor® conjugate (bottom) measured at an absorbance of 568 nm. X-axis is time (minutes); y-axis is UV absorbance (arbitrary units) of eluting compound.

References

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