## A Concise Synthesis of the Pennsylvania Green Fluorophore and Labeling of Intracellular Targets with *O*<sup>6</sup>-Benzylguanine Derivatives

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## ABSTRACT



We report improved syntheses of the Pennsylvania Green and 4-carboxy-Pennsylvania Green fluorophores; the latter compound was prepared from methyl 4-iodo-3-methylbenzoate in a three-pot process (32% overall yield). Chinese hamster ovary cells expressing *O*<sup>6</sup>-alkylguanine-DNA alkyltransferase fusion proteins were treated with Pennsylvania Green and Oregon Green linked to *O*<sup>6</sup>-benzylguanine (SNAP-Tag substrates). Analysis of living cells by confocal microscopy revealed that Pennsylvania Green derivatives exhibit substantially higher cell permeability than analogous Oregon Green-derived molecular probes.

Fluorescent molecular probes are powerful tools for studies of biological systems. Structurally diverse fluorophores, including small molecules, intrinsically fluorescent proteins, and semiconductor nanoparticles, have been particularly useful for investigating the localization and function of proteins in cells.<sup>1</sup> Among the small molecules, derivatives of fluorescein (**1**, Figure 1), a classic green fluorophore,<sup>2</sup> have been especially important. Some fluorescein derivatives such as 5- and 6-carboxyfluorescein can be readily synthesized<sup>3</sup> and exhibit high quantum yields in neutral and basic aqueous solutions (quantum yield of fluorescein = 0.925 in 0.1 N aqueous NaOH<sup>4</sup>). However, the high  $pK_a$  (~6.4)<sup>5</sup> of fluorescein and its 5- and 6-carboxy derivatives renders the quantum yield of these dyes quite sensitive to pH fluctuations observed under physiological conditions such as the environment in acidic endosomes of mammalian cells (pH ~5-6.5). Other limitations of many fluorescein derivatives include a relatively high susceptibility to photobleaching<sup>6</sup> and limited cell permeability<sup>7</sup> due to the predominant

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Figure 1. Structures of fluorophores and molecular probes in ionization states observed at physiological pH.

dianionic charge state of **1** at physiological pH (7.4).<sup>8</sup> The cell permeability of fluorescein can be improved by protection as a diester, but this modification adds synthetic complexity, eliminates fluorescence, and requires that intracellular esterases unmask the fluorophore.<sup>7</sup>

We recently reported the synthesis of a more hydrophobic, less pH-sensitive, and more photostable analogue of fluorescein termed Pennsylvania Green (2, Figure 1).<sup>9</sup> This new fluorophore and its 4-carboxy derivative (3) incorporate structural elements from the previously reported fluorophores Oregon Green<sup>5</sup> and Tokyo Green.<sup>10</sup> Oregon Green is a more acidic and photostable 2',7'- difluoro derivative of fluorescein first described by Haugland and co-workers. By contrast, Tokyo Green is a more hydrophobic analogue of fluorescein, more recently reported by Urano, Nagano, and co-workers, that replaces the carboxylate with a methyl group. By combining these structural features, Pennsylvania Green and derivatives represent potentially valuable tools for the construction of molecular and cellular probes. However, our previously reported 10-step synthesis of 4-carboxy-Pennsylvania Green (3), from 1,2,4-trifluoro-5-nitrobenzene in 16% overall vield, limited the availability of this material. To help overcome this limitation, we report here a three-pot synthesis of 4-carboxy-Pennsylvania Green (3) that affords a 32% overall yield, employs less expensive methyl 4-iodo-3methylbenzoate as the starting material, and allows preparation of gram quantities of the fluorophore.

To further characterize the biological properties of derivatives of Pennsylvania Green, fluorescent molecular probes 4 and 5 derived from  $O^6$ -benzylguanine (Figure 1) were investigated.  $O^6$ -Benzylguanine derivatives (SNAP-Tag substrates) are useful probes of cellular biology because they selectively alkylate the DNA repair protein  $O^6$ -alkylguanine-DNA alkyltransferase (AGT), enabling covalent labeling of AGT fusion proteins (SNAP-Tag fusion proteins).<sup>11</sup> The native human AGT protein is a monomeric protein of 207 amino acids that reacts with aberrant  $O^6$ -alkyl guanine residues to repair this mutagenic lesion in DNA. As shown in Figure 2 (panel A), X-ray crystallography has illuminated



**Figure 2.** X-ray structure of human AGT bound to  $O^6$ -methylguanine on DNA (panel A, pdb code 1T38) and mechanism of covalent labeling of AGT fusion proteins with  $O^6$ -benzylguanine-fluorophores (panel B).

the molecular details of interactions of AGT with modified guanine residues on the natural DNA substrate.<sup>12</sup> Nucleophilic attack of cysteine-145 of human AGT on alkylguanine derivatives repairs these modified guanines by transferring the alkyl group to cysteine and liberating free guanine (Figure 2, panel B).<sup>13</sup> In the absence of AGT,  $O^6$ -benzylguanine derivatives are stable under physiological conditions, and this approach can be used to selectively label AGT fusion proteins with fluorophores and other probes linked to  $O^6$ -benzylguanine. This technology has been improved and generalized by construction of mutants of AGT that increase the specificity of AGT for  $O^6$ -benzylguanines and that eliminate

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the DNA binding activity of this protein to facilitate studies of AGT fusion proteins localized outside the nucleus.<sup>14</sup> This method has been used to fluorescently label subcellular compartments in mammalian cells,<sup>15</sup> detect AGT fusion proteins in SDS-PAGE gels,<sup>16</sup> and immobilize proteins on solid support.<sup>17</sup> Other methods for site-specific labeling of proteins with fluorophores have also been reported.<sup>18–24</sup>

5- and 6-carboxyfluorescein and related compounds are typically synthesized by condensation of 1,3-dihydroxybenzene (resorcinol) with 1,2,4-benzenetricarboxylic anhydride (4-carboxyphthalic anhydride) under acidic conditions at relatively high temperatures.<sup>25</sup> To prepare pure isomers of 5- and 6-carboxyfluorescein on large scale, Burgess and coworkers reported the synthesis of isomeric methanesulfonic esters that can be readily separated by recrystallization.<sup>3</sup> Alternatively, Van Vranken and co-workers demonstrated that the fluorophore scaffold commonly used in metalsensitive fluorescein derivatives (fluo indicators) can be constructed by condensation of resorcinol with an aldehyde rather than an anhydride in the presence of dilute methanesulfonic acid, followed by oxidative cyclization with DDQ.<sup>26</sup> These milder reaction conditions avoid the generation of isomers and have the potential to allow access to a broader range of fluorophores.

Based on these precedents, we investigated the condensation of *o*-methylbenzaldehyde (6) with 4-fluororesorcinol (7) as a concise method to synthesize Pennsylvania Green (2). 4-Fluororesorcinol is commercially available, but we typically prepare it on a 5 gram scale using the two-step method of Vij and Shreeve<sup>27</sup> starting with 1,3-dimethoxybenzene and Selectfluor. As shown in Scheme 1, by condensing 6 with 7 in the presence of 9% methanesulfonic acid, triarylmethane 8 could be isolated in 83% yield. Initial attempts to prepare Pennsylvania Green (2) by oxidative cyclization with DDQ in AcOH/benzene afforded 2 in 28% yield. Further optimization revealed that treatment of 8 with excess *p*-TsOH in refluxing toluene, a method previously reported by Thongpanchang for synthesis of oxa-helicenes,<sup>28</sup> was more effec-

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B) (A) HO OHOH сно HO мe p-TsOH 2 (37%)MeSO<sub>3</sub>H toluene Me 6 8 CH<sub>2</sub>Cl<sub>2</sub> reflux Et<sub>2</sub>O (83%) OH OH (B) HC



tive, providing 2 in 37% yield. To prepare 4-carboxy-Pennsylvania Green (3), commercially available methyl 4-iodo-3-methylbenzoate (9) was employed (Scheme 1, panel B). This aryl iodide (9) was subjected to halogen-metal exchange using *i*-PrMgCl and subsequently formylated with DMF in 95% yield.<sup>29</sup> The resulting aldehyde (10) was condensed with 4-fluororesorcinol (7) in 9% methanesulfonic acid to afford triarylmethane 11 in 88% yield. Treatment of 11 with DDQ or chloranil did not afford the cyclic product, presumably due to the influence of the electron-withdrawing ester substituent. However, heating to reflux in toluene with excess *p*-TsOH sucessfully promoted cyclization; subsequent base-promoted hydrolysis of the methyl ester in the same pot yielded the desired product (3) in 38% yield. Attempts to further improve the yield by addition of Lewis acids analogous to the approach reported by Suzuki<sup>30</sup> decreased the yield or impeded purification of the final product.

Molecular probes 4 and 5 were prepared by acylation of commercially available  $O^6$ -[4-(aminomethyl)benzyl]guanine (14)<sup>11</sup> with succinimidyl ester derivatives of 4-carboxy-Pennsylvania Green (12) and 5-carboxy-Oregon Green (13) (Scheme 1). These structurally similar AGT substrates were prepared to examine differences in cellular permeability resulting from substitution of the carboxylate of 5 with the methyl substituent of 4. Previous studies of AGT fusion proteins expressed within mammalian cells have reported that the efficiency of labeling is critically dependent on the cell permeability of the substrate.<sup>15</sup> To confirm that differences in cellular labeling do not result from changes in physico-

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chemical properties, the maximal absorbance (abs) and emission (em) wavelengths, molar extinction coefficients ( $\epsilon$ ), and quantum yields ( $\Phi$ ) of compounds 1–5 were determined<sup>31</sup> (see the Supporting Information for details), and these values are listed in Table 1. We next examined the

**Table 1.** Physicochemical Properties of Fluorophores andFluorescent Probes<sup>a</sup>

compound	abs/em (nm)	$\stackrel{\epsilon}{(\mathrm{M}^{-1}\mathrm{cm}^{-1})}$	$\Phi$ (pH)
fluorescein (1)	490/514	77 000	0.925 (9)
Pennsylvania Green (2)	494/514	82 000	0.91(7.4)
4-carboxy	494/515	$62\ 000$	0.89(7.4)
Pennsylvania Green (3)			
O6-benzylguanine-	494/515	$58\ 000$	0.92(7.4)
Pennsylvania Green (4)			
O6-benzylguanine-	494/522	$73\ 000$	0.97(7.4)
Oregon Green (5)			

 $^{a}$ Values for fluorescein (1) were previously reported.<sup>4,8</sup> Values for compounds 2–5 were determined in phosphate-buffered saline (PBS) at pH 7.4.

ability of probes **4** and **5** to efficiently label intracellular targets in Chinese hamster ovary-K1 (CHO) cells. These cells were transiently transfected with commerically available plasmid vectors (Covalys Biosciences) encoding AGT fusion proteins that become targeted to distinct subcellular locations. To image the plasma membrane, AGT was expressed fused to a C-terminal CAAX peptide sequence from a human Ras protein, which is associated with the inner leaflet of the cellular plasma membrane.<sup>14</sup> Conversely, AGT was expressed fused to the C-terminus of the human histone 2B (H2B) protein to promote localization in the cell nucleus.<sup>15</sup>

CHO cells transiently expressing AGT fusion proteins were treated with molecular probes and analyzed by confocal laser scanning microscopy. As shown in Figure 3, only the Pennsylvania Green derivative 4 (5  $\mu$ M, 1 h treatment) was capable of clearly labeling the cellular plasma membrane upon expression of the AGT-CAAX fusion protein. Under identical conditions, the Oregon Green probe 5 showed only punctate fluorescence throughout the cytoplasm of the subset of transfected cells (compare panels A and C of Figure 3). Transient transfection of CHO cells with the nuclear localized AGT-H2B fusion protein revealed strong nuclear fluorescence upon treatment with 4 (5  $\mu$ M, 1 h) but only weak nuclear fluorescence upon treatment with 5 under these conditions (compare panels B and D of Figure 3).

Our results demonstrate that the subtle molecular substitution of the carboxylate of Oregon Green for the methyl group of Pennsylvania Green can profoundly enhance the cellular permeability and consequent biological activity of  $O^6$ benzylguanine derivatives. The concise synthesis of Penn(A) Penn Green probe 4 / AGT-CAAX



Fluorescence DIC + Fluorescence

**Figure 3.** Confocal laser scanning and differential interference contrast (DIC) micrographs of CHO cells expressing AGT fusion proteins and treated with probes 4 and 5. CAAX: C-terminal peptide derived from the Ras protein that associates with the inner leaflet of the plasma membrane. H2B: nuclear-localized histone 2B protein. Molecular probes were added at 5  $\mu$ M for 1 h at 37 °C. Scale bar = 10  $\mu$ M.

sylvania Green and derivatives reported here has the potential to facilitate studies of cellular biology requiring cell permeable and pH-insensitive green fluorescent molecular probes.

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**Supporting Information Available:** Experimental procedures and characterization data for new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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