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Bioorganic & Medicinal Chemistry Letters 14 (2004) 2725–2728
Bioorganic & Medicinal Chemistry Letters

Synthesis and characterization of bifunctional probes for the specific labeling of fusion proteins

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Received 19 October 2003; revised 17 March 2004; accepted 26 March 2004

Abstract—Labeling proteins with synthetic probes is important for studying and characterizing protein function. We have recently introduced a general method for the specific in vivo and in vitro labeling of fusion proteins that is based on the reaction of O^6-alkylguanine-DNA alkyltransferase (AGT) with O^6-benzylguanine derivatives. Here we report two complementary routes for the synthesis of O^6-benzylguanine derivatives, which allow for the labeling of AGT fusion proteins with bifunctional synthetic probes and demonstrate the specific labeling of AGT fusion proteins with these probes. These molecules should become useful tools for various applications in functional proteomics.

Studying proteins often relies on providing the protein of interest with a unique property that allows either for purification from complex mixtures or for characterization in functional assays in vivo or in vitro. Usually, the unique property is genetically encoded by expressing the protein of interest as a fusion with an additional polypeptide such as the hexa-histidine tag for protein purification or with an autofluorescent protein for studying protein function in living cells.[1,2] Alternatively, the protein of interest can be chemically labeled with a synthetic probe that possesses the desired unique property.[3,4] While a wide variety of synthetic probes with interesting properties for the studying of protein function are available, the specific labeling of the protein of interest remains a challenge.[3] We have recently introduced a method that allows for the specific labeling of fusion proteins of O^6-alkylguanine-DNA alkyltransferase (AGT) with synthetic probes and which is well suited for applications in vivo and in vitro.[5] The specific labeling of AGT is based on its reaction with cell-permeable O^6-benzylguanine (BG) derivatives of type **1**, leading to the transfer of an appropriate label to a reactive cysteine of AGT (Fig. 1A). The velocity of the reaction between AGT and **1** is not significantly influenced by the nature of the label, allowing the transfer of a wide variety of different probes to AGT fusion proteins. Furthermore, we have recently shown that AGT fusion proteins can be specifically and covalently immobilized on surfaces displaying BG.[6] An important extension of this approach for a variety of applications would be the use of BG derivative of type **2** (Fig. 1B), leading to the labeling of AGT fusion proteins with two different probes.[7] For example, the use of BG derivative **3** (Fig. 1C) would lead to the labeling of AGT fusion proteins with the spectroscopic probe fluorescein and the affinity label biotin, two labels that are employed in a variety of functional assays. BG derivatives **4** and **5** (Fig. 1C) would lead to the labeling with two different affinity probes: dinitrophenol and biotin in the case of **4**, and digoxigenin and biotin in the case of **5**. A possible application for bifunctional probes such as **4** or **5** would be their use in tandem affinity purifications of proteins out of complex mixtures using the immobilized corresponding antibody and avidin.[8]

As starting point for the synthesis of bifunctional BG derivatives we chose **6** and **7**, in which orthogonally protected L-lysine derivatives **9**[9–11] or **10** are linked to the previously described BG derivative **8** (Fig. 2). Bifunctional labels of the type **2** can then be synthesized by successive couplings of the desired probes to one of the two deprotected amino functionalities of **6** (Scheme 1) or **7** (Scheme 2). Two lysine derivatives with different combinations of orthogonal protection groups were prepared as the synthesis of bifunctional labels with

Keywords: Substrate analogs; Proteomics; Protein labeling.
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doi:10.1016/j.bmcl.2004.03.078

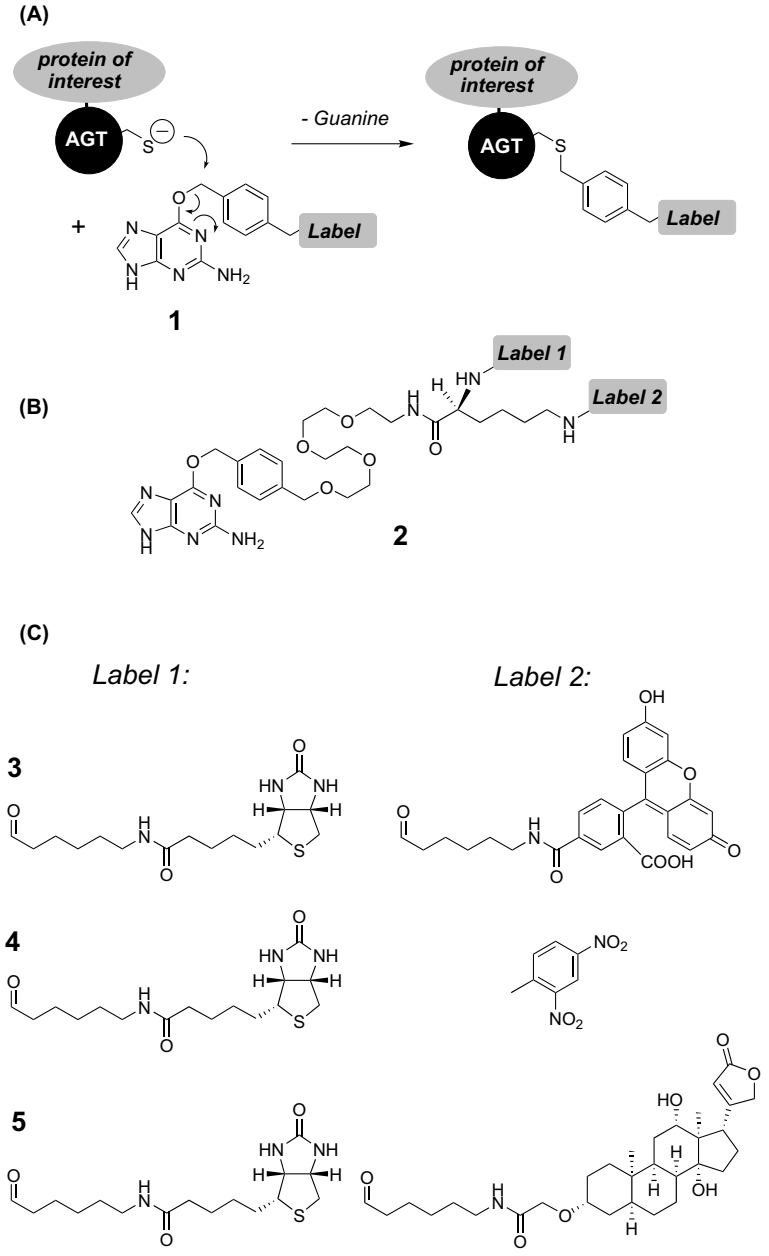


Figure 1. Labeling of AGT fusion proteins with synthetic molecules. (A) Mechanism of labeling reaction; (B) general structure of bifunctional BG derivatives; (C) bifunctional BG derivatives **3**–**5** used for the labeling of AGT fusion protein.

different probes might require different conditions for deprotection, although for the synthesis of the bifunctional derivatives presented here a single set of protecting groups would have been sufficient. Compounds **6** and **7** were synthesized by condensing **8** with the corresponding orthogonally protected lysine derivatives **9** or **10** (Schemes 1 and 2). Concerning α-azido-ε-N-Fmoc-lysine **9**, the use of an azide to mask the α-amino group of lysine was described earlier and **9** was prepared from commercially available Fmoc-lysine using published procedures.[10] For the synthesis of **3**, the α-azido group of **6** was reduced under neutral conditions with trimethylphosphine in aqueous 1,4-dioxane, yielding the corresponding amine **11** (Scheme 1). Compound **11** was reacted with N-(+)-biotin-6-amino-caproic acid N-succinimidyl ester to give **12**, followed by Fmoc deprotection with diethylamine to yield intermediate **13**. Fluorescein-5(6)-carboxamidocaproic acid N-succinimidyl ester was reacted with the free ε-amino group of **13**, leading to the bifunctional substrate **3**. Synthesis of the bifunctional labels **4** and **5** started from the fully protected **7**, which was prepared by condensing the commercially available α-N-Fmoc-ε-N-Dde-lysine **10** with **8** under standard peptide coupling conditions (Scheme 2).[12] Fmoc deprotection with diethylamine followed by coupling of N-(+)-biotin-6-aminocaproic acid N-succinimidyl ester yielded intermediate **15**. The Dde-protecting group was cleaved by using a 4% hydrazine solution, yielding intermediate **13** with a primary amine for further derivatizations. For the synthesis of **4**, **13** was coupled to 2,4-dinitrofluorobenzene. The reaction of digoxigenin-3-O-methylcarbonyl-ε-amino-

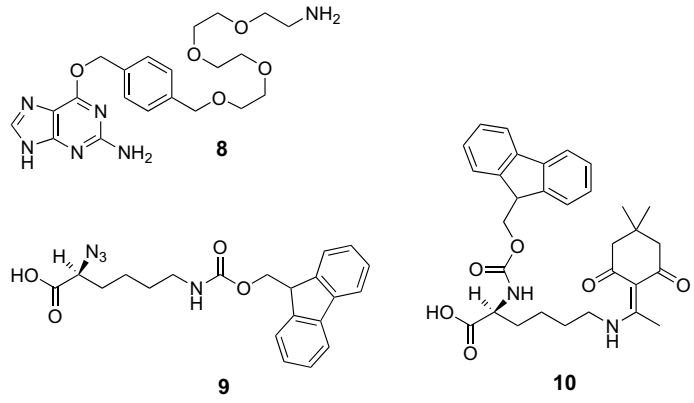
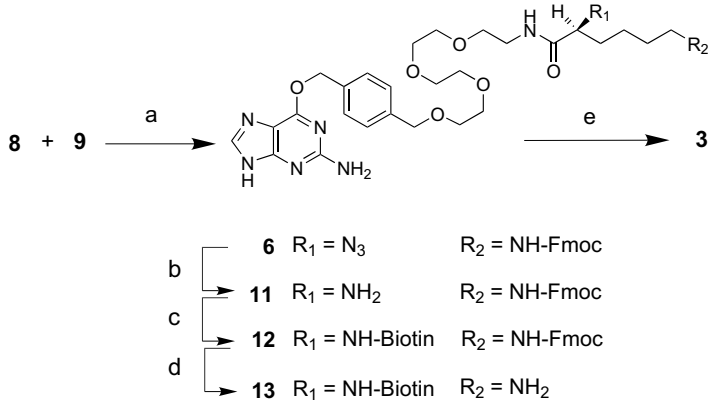
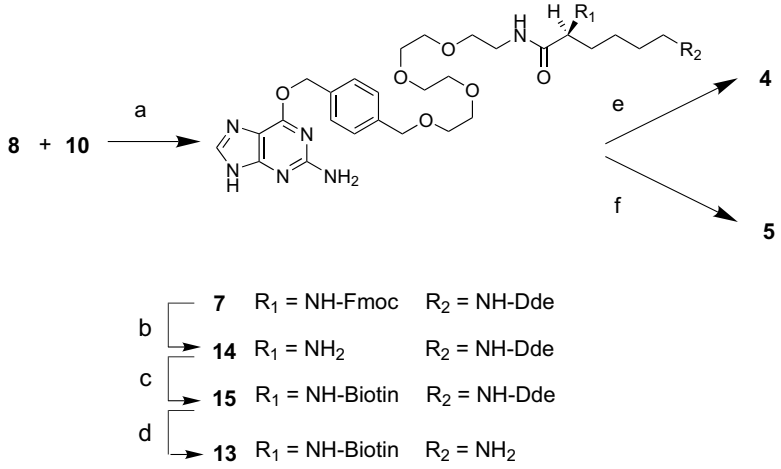


Figure 2. Structures of (**8**)–(**10**).



Scheme 1. Synthesis of the bifunctional BG derivative **3**. Reagents and conditions: (a) EDC, HOBT (1 M in NMP) (2.5 equiv), DMF, 2 h, 57%; (b) 1,4-dioxane/H_2O (8:1), trimethylphosphine (1 M in THF), 1 h, 95%; (c) N-(+)-biotin-6-amino-caproic acid N-succinimidyl ester, DIPEA, DMF, 40 min, 71%; (d) diethylamine, DMF, 40 °C, 1.5 h, quant.; (e) fluorescein-5(6)-carboxamidocaproic acid N-succinimidyl ester, DIPEA, DMF, 40 min, 67%.



Scheme 2. Synthesis of the bifunctional BG derivatives **4** and **5**. Reagents and conditions: (a) EDC, HOBT (1 M in NMP) (2.5 equiv), DMF, 2 h, 64%; (b) diethylamine, DMF, 40 °C, 1.5 h; (c) N-(+)-biotin-6-amino-caproic acid N-succinimidyl ester, DIPEA, DMF, 2 h, 37% over two steps; (d) hydrazine (4% in DMF), 20 min, then quenching with acetone; (e) 2,4-dinitrofluorobenzene, triethylamine, 45 °C, 2 h, 75%; (f) digoxygenin-3-oxymethyl-carbonyl)-6-aminocaproic acid N-hydroxysuccinimide ester, triethylamine, 40 °C, 2 h, 45%.

caproic-acid-N-hydroxy-succinimide with the free amino group of **13** yielded compound **5**.

To demonstrate that the bifunctional probes **3**–**5**[13] are substrates of AGT fusion proteins and that after the

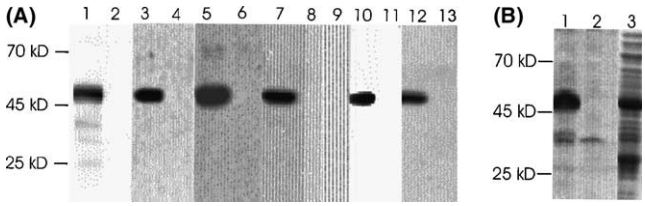


Figure 3. (A) AGT–GST fusion protein (2 μM) was labeled with the respective bifunctional probe (15 min, rt). Lane 1: GST–AGT labeled with **3** (25 μM), developed with neutravidin; lane 2: GST–AGT without label as negative control, developed with neutravidin; lane 3: GST–AGT labeled with **5** (65 μM), developed with neutravidin; lane 4: GST–AGT without label, developed with neutravidin; lane 5: GST–AGT labeled with **4** (150 μM), developed with neutravidin; lane 6: GST–AGT without label, developed with neutravidin; lane 7: GST–AGT labeled with **3**, developed with anti-fluorescein antibody; lane 8: AGT–GST without label, developed with anti-fluorescein antibody; lane 9: GST–AGT labeled with **3**, developed with anti-digoxygenin antibody; lane 10: GST–AGT labeled with **4**, developed with anti-DNP antibody; lane 11: GST–AGT without label, developed with anti-DNP antibody; lane 12: GST–AGT labeled with **5**, developed with anti-digoxygenin antibody; lane 13: GST–AGT without label, developed with anti-digoxygenin antibody; (B) lane 1: cell extract of *E. coli* expressing GST–AGT fusion protein, labeled with substrate **5**, Western probed with anti-digoxygenin antibody; lane 2: cell extract of *E. coli* expressing only GST after incubation with bifunctional probe **5**, Western probed with anti-digoxygenin antibody; lane 3: SDS gel from cell extract, stained with Coomassie.

reaction the probes can be recognized by other proteins, we incubated **3**–**5** with a fusion protein of AGT with glutathione S-transferase (GST–AGT) and analyzed the labeling by probing Western blots of these samples with appropriate antibodies or streptavidin (Fig. 3). In these experiments, 30 μL of a solution of the fusion protein (2 μM) were incubated with the bifunctional probes (25 μM for substrate **3**, 150 μM for substrate **4** and 65 μM for substrate **5**) for 15 min at room temperature before the reaction was analyzed by Western blotting. As in earlier experiments, the labeling of the fusion protein was nearly quantitative (data not shown).[5] The corresponding data show that all bifunctional probes are accepted as substrates of GST–AGT and that each individual probe can be recognized by either specific antibodies or streptavidin. Furthermore, GST–AGT labeled with biotin and fluorescein using the bifunctional substrate **3** was not recognized by an anti-digoxiginin antibody (Fig. 3, lane 9). We also measured the absorption and emission spectra of GST–AGT after incubating the fusion protein with **3** and removing excess label through gel filtration. The measured maxima for absorption and emission of the labeled protein of 498 and 526 nm, respectively, are in agreement with the corresponding values of fluorescein. To demonstrate the specificity of the reaction of AGT fusion proteins with bifunctional labels of type **2**, we incubated **5** with cell extract of *E. coli* expressing GST–AGT and examined the specificity of the labeling by probing Western blots of the sample with an anti-digoxigenin antibody (Fig. 3). This experiment shows that the bifunctional probes can be used for the specific labeling of AGT fusion proteins in complex mixtures.

In summary, we present here two routes for the facile preparation of bifunctional BG derivatives of the type **2**, which can be used for the selective labeling of AGT fusion proteins. The developed chemistry should allow the synthesis of BG derivatives displaying various combinations of interesting probes, including cross-linkers, photosensitizers or spectroscopic probes, and should make bifunctional BG derivatives useful tools for applications in functional proteomics.

Acknowledgements

This project was funded by the Swiss National Science Foundation (2100-067983). I.S. was supported by a fellowship of the Fonds der Chemischen Industrie (FCI). We thank Nils Johnsson for valuable advice and discussions.

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