Specific Labeling of Polypeptides at Amino-Terminal Cysteine Residues Using Cy5-benzyl Thioester

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Even for moderately sized proteins, the multiple occurrence of cysteine and lysine residues often prevents the specific labeling of polypeptides with a single probe. To increase specificity, a method was developed to convert the commonly available succinimidyl esters of fluorescent dyes into benzyl thioesters via trimethyl aluminum-activated benzyl mercaptan. The thioester can then be reacted very specifically with polypeptides containing an N-terminal cysteine residue, forming a stable amide bond, analogous to the native chemical ligation of peptide fragments. Both reaction steps are easy to perform and proceed to high yields. The practicability of the approach was demonstrated using the popular cyanine dye Cy5 and a soluble peptide, and it is expected to be applicable to a wide range of succinimidyl esters and both chemically and recombinantly synthesized proteins. The method should dramatically facilitate the preparation of proteins for experiments requiring exact positioning of labels, for instance, Förster resonance energy transfer studies.

INTRODUCTION

A general problem in the specific conjugation of chromophores or other molecules to polypeptides is the limitation to sulfhydryls and amines as suitable reactive groups. Therefore, most commercially available fluorophores can be obtained with functionalities that allow specific labeling of cysteine or lysine residues and the free amine at the N-terminus (Haugland, 2001). However, except for small peptides, the statistical and therefore often multiple occurrence of cysteine and lysine residues in one polypeptide prevents the specific attachment of just one label to a protein. For some applications, like in vivo imaging, the degree of labeling is only of secondary importance, although it is well-known for several fluorophores that severe fluorescence quenching can occur if multiple sites on one protein are modified (Hermanson, 1996; Gruber et al., 2000). For an increasing number of experimental approaches, specificity is strictly required, for instance the use of fluorophores for FRET[1] or polarization studies both in ensemble and single molecule experiments (Weiss, 1999). So far, the only solution to this problem has been solid-phase peptide synthesis followed by labeling of the polypeptide before side chain deprotection, sometimes in combination with native chemical ligation of peptide fragments (Deniz et al., 2000). A general approach for the specific attachment of single labels to recombinantly expressed proteins has not been available, which sometimes makes extensive sequence changes necessary to eliminate cysteine and lysine residues, thus potentially interfering with the stability and function of the protein. Here we present a simple protocol for the synthesis of thioester derivatives starting from succinimidyl esters, the function most commonly employed for the specific labeling of amino groups. These thioester derivatives can then be used to label polypeptides with an aminoterminal cysteine residue in a highly specific reaction analogous to the native chemical ligation of peptides (Dawson and Kent, 2000). In this case, the necessary sequence changes are limited to the introduction of a cysteine residue at the amino terminus.

EXPERIMENTAL PROCEDURES

Materials. The peptide Cys-(Ala-Gly-Gln)$_5$-Trp-CONH$_2$ (calculated average mass 1587.6 Da, ESI-MS, positive mode, observed average mass 1587.3 ± 0.3 Da) was obtained from California Peptide Research (Napa, CA). The carboxy terminal Trp residues was included to provide an intrinsic chromophore for accurate peptide concentration determination. The control peptide Ala-Gly-Gln-Lys-Cys-Lys-Ala-Gly-Gln-Trp was synthesized using standard FastMoc chemistry with a Model 433A peptide synthesizer (Applied Biosystems) on Fmoc-Trp-resin (Biosearch Research Chemicals). After cleavage, the raw material was purified by preparative reversed phase HPLC. Fractions containing the pure peptide as confirmed by electrospray ionization mass spectroscopy (calculated mass 1075.5 Da, ESI-MS, positive mode, observed mass 1075.5 ± 0.3 Da) were lyophilized and stored at −20 °C. Cy5-succinimidyl ester was obtained from Amersham Pharmacia Biotech, hydroxylamine from Pierce. All other chemicals and solvents were from Aldrich (Milwaukee, WI). All reactions were performed at room temperature. Exposure of dye solutions to bright light was avoided. A VirTis Benchtop 3.3 freeze-dryer with a condenser temperature of −105 °C was used for

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[1] Abbreviations: DMF, dimethylformamide; ESI-MS, electrospray ionization mass spectroscopy; FRET, Förster resonance energy transfer; FABMS, fast atom bombardment mass spectroscopy, TFA, trifluoroacetic acid; NHS, *N*-hydroxysuccinimide.

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lyophilization in order to minimize damage to the vacuum pump from uncondensed acetonitrile and other organic solvents.

Synthesis of Cy5-thioester. The following reactions were performed under a dry nitrogen atmosphere. It is advisable to use Hamilton syringes, ensure good ventilation, and avoid contact of the reaction mixture with water or oxygen. Benzyl mercaptan (117 μL, 1.0 mmol) was slowly added to a mixture of 0.5 mL of 2.0 M trimethylaluminum (1.0 mmol) in hexane and 2 mL of anhydrous dichloromethane cooled to 0 °C in a 4 mL glass reaction vessel while stirring. The reaction was allowed to warm to room temperature over a 20 to 30 min period. A 100 μL volume of this solution was added to 1 mg of Cy5-succinimidyl ester thoroughly dissolved in 50 μL of anhydrous DMF, yielding a clear colorless solution, and stirred for about 60 min. Solubilization of the dye in DMF at high concentration was slightly batch-dependent. Incomplete dissociation of the dye can be detected by a characteristic increase of the absorbance band at 600 nm. In this case, sonication should be continued until the absorbance spectrum of the monomeric dye is observed.

To avoid precipitation of the product under purification conditions, 20 μL portions of the reaction mixture were diluted 5-fold with DMF and added to 1 mL of a 10% acetonitrile/90% water mixture plus 0.1% TFA while stirring vigorously, centrifuged for 5 min at 10000*g* in a regular 1.5 mL polypropylene reaction vessel to clear the solution completely, and applied to a preparative reversed phase HPLC column. Fractions containing Cy5-thioester as detected by absorbance at 648 nm were combined, and the dye concentration was determined using the same molar extinction coefficient (ϵ = 250000 M^{-1} cm^{-1}) as published for the Cy5-monofunctional dye (Mujumdar et al., 1993). Portions corresponding to 10 to 100 nmol of Cy5-thioester were frozen in liquid nitrogen, lyophilized, and stored at −20 °C.

Reversed Phase HPLC. Analytical and preparative gradient HPLC were performed on a Waters 600E multisolvent delivery system equipped with a Waters 996 photodiode array absorbance detector. Preparative HPLC was run on a Vydac C4 column (214TP1022, 5 μm particle diameter, 22 × 250 mm) at a flow rate of 5 mL/min over 45 min. Analytical HPLC was performed on a Vydac C4 column (214TP54, 5 μm particle diameter, 4.6 × 250 mm) at a flow rate of 1 mL/min over 30 min. All runs used linear gradients of 0.1% TFA in 10% acetonitrile/90% water vs 0.1% TFA in 90% acetonitrile/10% water.

Peptide Labeling. The peptides were dissolved in buffer containing 6 M GdmCl, 100 mM sodium phosphate, pH 7.6, to a concentration of 1 to 2 mM, and 4% thiophenol was added. Peptide concentrations were determined using the absorbance of the single tryptophan residue present in the sequence (ϵ = 5690 M^{-1} cm^{-1} at 280 nm; Gill and von Hippel, 1989). Cy5-thioester was dissolved in DMF to a concentration of 20 mM, sonicated, and vortexed to completely dissolve the dye and then added to the peptide solution to give a final dye concentration equimolar with the peptide. The solution was vortexed to dissolve the thiol additive and then incubated at room temperature in the dark. The reactions were monitored by analytical reversed phase HPLC.

RESULTS AND DISCUSSION

The family of cyanine dyes (Mujumdar et al., 1993) has become one of the most frequently used groups of fluorophores in high sensitivity fluorescence detection and optical single molecule experiments due to their high extinction coefficients, high fluorescence quantum yields, and resistance to photobleaching. Especially Cy5 has attracted much attention due to its extremely long-wavelength fluorescence emission maximum at 667 nm, allowing excellent spectral separation in studies requiring multiccolor detection, e.g., single molecule fluorescence resonance energy transfer experiments (Ha, 2001). Currently, it is commercially available as a succinimidyl ester, which can be used for the modification of amines, and as a maleimide, which reacts with the sulfhydryl groups of cysteine residues. To increase the selectivity of protein labeling even further, we decided to devise a method that allows modification of a single position in a polypeptide chain. To this end, the succinimidyl ester of Cy5 was first converted to a benzyl thioester, which can then undergo a highly specific conjugation reaction with an N-terminal cysteine residue in a polypeptide chain.

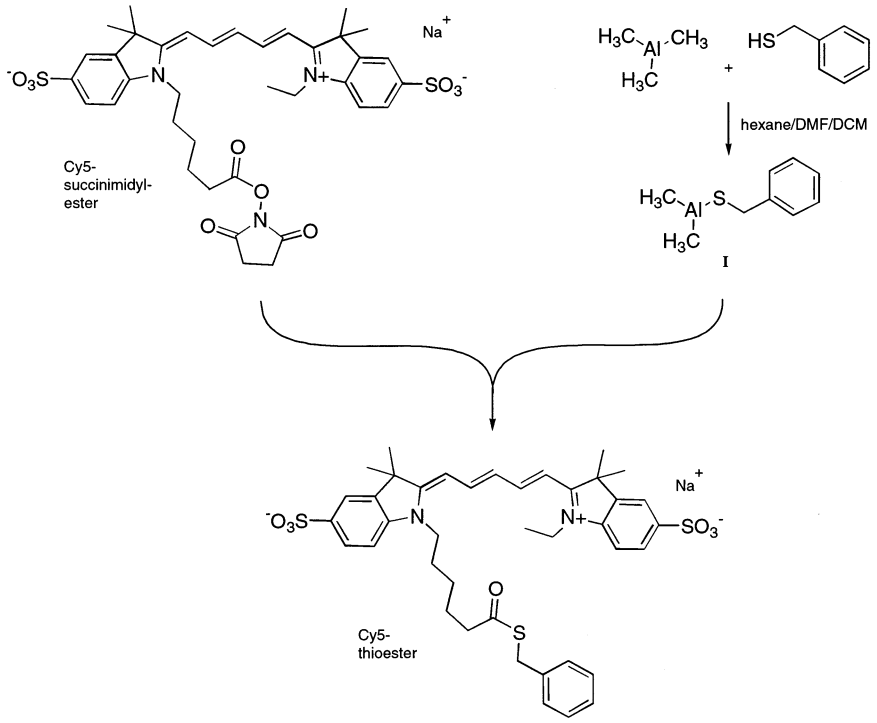
Synthesis of Cy5-thioester. The direct formation of Cy5-thioester from the succinimidyl ester and an aromatic thiol like benzyl mercaptan proceeds to only very low yields (unpublished observation) and can thus not be used for an efficient synthesis. Therefore, a two-step reaction was used to synthesize Cy5-thioester starting from the Cy5-succinimidyl ester (or N-hydroxy succinimide/NHS ester) (Scheme 1). Initially, the aluminum reagent **I** was prepared from an equimolar mixture of trimethylaluminum and benzyl mercaptan in a protocol similar to the one described by Hatch and Weinreb in 1977. These aluminum compounds are known to react with a variety of esters at room temperature to produce the respective thioesters in good yield (Corey and Beames, 1973; Cohen and Gapinski, 1978) and can be used without further purification. It was therefore attempted to use this very simple procedure to generate the benzyl thioester of Cy5.

Surprisingly, incubation of Cy5-succinimidyl ester with the reaction mixture containing compound **I** resulted in a colorless solution, possibly via interaction of the chromophore with the aluminum compounds. It regained its deep blue color after dilution in aqueous solutions or acetonitrile/water mixtures, indicating a reversible association with some component of the reaction mixture. Analytical reversed phase HPLC indicated quantitative conversion of the succinimidyl ester after 60 min. The product eluted as a single peak with a clear shift to larger elution volume (Cy5-succinimidyl ester: 14.5 mL, Cy5-thioester: 16.5 mL), as would be expected from the presence of the more hydrophobic benzyl group instead of the *N*-hydroxysuccinimidyl group. The yield of thioester as calculated from the integrated absorbance signal at 648 nm was 95 ± 5%. The calculated molecular mass of the Cy5-benzyl thioester of 762.2 Da was confirmed by ESI-MS (positive mode, $[M - H]^+$ 763.3 ± 0.2 Da) and FABMS (negative mode, $[M - H]^-$ 761.2 ± 0.2 Da). Lyophilized product retained its reactivity for months if stored at −20 °C.

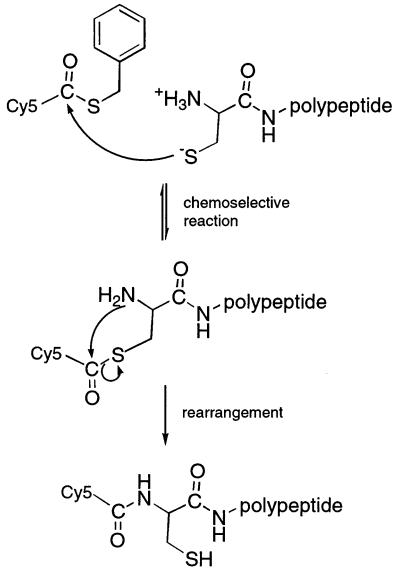
Spectroscopically, no significant differences of Cy5-thioester compared to the succinimidyl ester were detected. The absorbance maximum was at 648 nm for both derivatives, and the fluorescence quantum yield was unaltered in the thioester.

Peptide Labeling. The labeling approach described here makes use of the same chemistry as the method known as native chemical ligation (Scheme 2; Dawson et al., 1994; Dawson and Kent, 2000), which was developed for the synthesis of proteins from smaller, in particular chemically synthesized peptides. Exchange of a thioester with the thiol moiety of the N-terminal cysteine side chain gives a thioester-linked intermediate

Scheme 1. Conversion of Cy5-succinimidyl Ester to Cy5-thioester via Activation of Benzyl Mercaptan with Trimethylaluminum



Scheme 2. Probable Mechanism of the Peptide Labeling Reaction in Analogy to Native Chemical Ligation[a]



[a] Adapted from Dawson and Kent, 2000.

as the initial covalent product in a reversible reaction. Without change in the reaction conditions, a rapid intramolecular reaction occurs, forming the thermodynamically favored amide bond at the ligation site. This second step is irreversible under the conditions used, leading to the formation of the ligation product in high yield. Assuming that the mechanism of this reaction is not influenced significantly by the structure of the component containing the thioester, the reaction was adapted for the "ligation" of a polypeptide with the benzyl thioester of Cy5.

To test the reactivity of the Cy5-thioester in this type of reaction, a chemically synthesized model peptide with an N-terminal cysteine residue was used (see Experimental Procedures), and the reaction was carried out under essentially the same conditions as described for the native chemical ligation of peptides. The reaction is usually performed in 6 M GdmCl, under strongly denaturing conditions, which causes the reactivity of the N-terminal cysteine residue to be independent of the three-dimensional structures of the polypeptides in their native state. Additionally, 4% thiophenol were included in the reaction mixture. This has been shown to accelerate the reaction and increase reaction yields due to the formation of the more reactive phenyl thioester through in situ transesterification (Dawson et al., 1997). Additionally, excess thiol keeps cysteine side chains reduced and catalyzes the reversal of unproductive thioester formation. The advantage of using the less activated benzyl thioester as the primary component in the reaction mixture is its lower susceptibility to hydrolysis during storage and handling.

Analytical HPLC showed the emergence of two new components during the course of the labeling reaction eluting at volumes of 13.8 and 15.8 mL, which were identified by mass spectrometry as the labeled peptide (calculated average mass: 2226.5 Da, ESI-MS positive mode: observed average mass 2226.2 ± 0.3 Da) and Cy5-phenyl thioester (calculated mass: 748.2 Da, ESI-MS positive mode: $[M - H]^+$ 749.2 ± 0.2 Da), respectively. Figure 1 shows the kinetics of their formation at a peptide and Cy5-thioester concentration of 1.4 mM. The appearance of the signal corresponding to the labeled peptide exhibits a slight lag phase, during which a steady-state level of phenyl thioester builds up. This supports the concept of in situ transesterification of Cy5-benzyl thioester to the more reactive Cy5-phenyl thioester preceding the ligation reaction as described above. Under these conditions, a final Cy5-peptide yield of about 37% was reached after 24 h (Figure 1). At peptide and Cy5-thioester concentrations of about 2 mM, final yields of up to 68% labeled peptide were achieved. It would obviously be expected that the equilibrium can be shifted

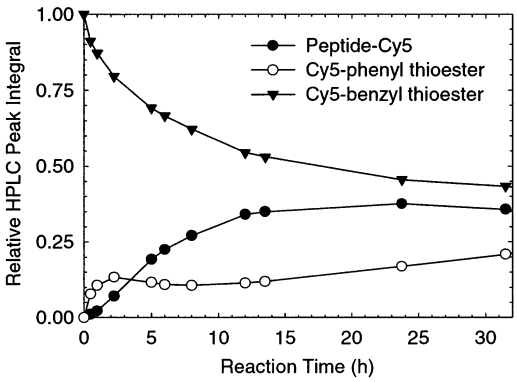


Figure 1. Kinetics of peptide labeling with Cy5-benzyl thioester at 20 °C in 6 M GdmCl, 100 mM sodium phosphate, pH 7.6, 4% thiophenol. Both peptide and Cy5-thioester concentration were 1.4 mM. Analytical HPLC runs were monitored by absorbance at 648 nm, all eluting peaks were integrated, and the relative fraction of the individual species was calculated, assuming equal extinction coefficients.

toward the products even further by increasing the concentrations of the reactants. Concentrated solutions of GdmCl (6 M) are very good solvents for unfolded proteins, allowing concentrations of up to 100 mg/mL (Rudolph et al., 1997). The solubility of the Cy5-thioester is more limited under these conditions. Even at 1 mM, it needs to be thoroughly predissolved in DMF, and a concentration of about 10% DMF must be present in the reaction mixture to avoid precipitation of the dye. At higher DMF concentrations, analytical HPLC indicated the accumulation of an unidentified side product heterogeneous in molecular mass at an elution volume of about 14.4 mL in analytical HPLC, which limits the concentration of DMF that can be used. Depending on the availability of the individual reaction components, increasing the protein concentration may thus be the more practicable approach.

After purification of the Cy5-peptide conjugate by preparative reversed phase HPLC, the lyophilized powder was redissolved in 6 M GdmCl, 100 mM sodium phosphate buffer, pH 7. The spectroscopic properties of the labeled peptide were very similar to those of the unreacted succinimidyl ester and the benzyl thioester of Cy5. The absorbance maximum was at 648 nm in 6 M GdmCl, and at 649 nm after dilution to 100 mM GdmCl. Cy5 fluorescence was quenched by 10 to 20% after attachment to the peptide (depending on GdmCl concentration), possibly due to the proximity of the tryptophan residue in the peptide. Similar effects are well-known for a variety of fluorescent dyes upon attachment to proteins and are apparently due to charge-transfer interactions with aromatic amino acid residues (Watt and Voss, 1977). Altogether, the peptide labeled by the protocol described here has the same spectroscopic properties expected from Cy5 conjugates using other commonly available chemistries such as maleimides or succinimidyl esters.

To determine the degree of specificity of the reaction for aminoterminal Cys residues, we used the peptide Ala-Gly-Gln-Lys-Cys-Lys-Ala-Gly-Gln as a control, which contains a central Cys flanked by two Lysine residues. In this case, the Cys would also be expected to form a thioester derivative with Cy5, which could then possibly be attacked by the ϵ-amino groups of the neighboring lysines, which have a p*K* value very similar to that of the α-amino group of cysteine. In a control reaction of this peptide with Cy5-benzyl thioester set up analogous to the one described above, 49% of the control peptide were found to be derivatized by the dye after 24 h, as determined by analytical HPLC and mass spectrometry (ESI-MS positive mode: observed average mass 1714.3 $\pm$ 0.3 Da). However, the mass of the thioester and a potential amide derivative are indistinguishable (calculated average mass:1714.0 Da), necessitating further characterization of the linkage. After incubation of samples of the reaction diluted 10-fold with 50 mM hydroxylamine in 6 M GdmCl, 100 mM sodium phosphate, pH 7.6 for 5 h, no labeled peptide was detectable by HPLC or mass spectrometry. This shows that no amide bonds had been formed between the ϵ-amino group of the lysine residues and Cy5. Instead, only Cy5-hydroxylamine was found as a product (calculated mass: 671.0 Da, ESI-MS positive mode: $[M - H]^+$ 671.1 $\pm$ 0.2 Da), resulting from the reaction of both the peptide-Cy5 thioester and unreacted Cy5-benzyl or Cy5-phenyl thioester with hydroxylamine. In the case of the peptide labeled at the aminoterminal Cys, on the other hand, hydroxylamine did not cause removal of the dye, as expected for an amide linkage. All the Cy5-hydroxylamine formed in this reaction originated from unreacted Cy5-benzyl and Cy5-phenyl thioester. This result demonstrates the extremely high specificity of the labeling reaction for aminoterminal Cys residues and illustrates the important role of the geometric arrangement of the α-NH_2 moiety with respect to the thioester formed in the first reaction step (Dawson et al., 1994). Additionally, it shows that dye molecules bound to internal Cys residues via thioester bonds can be removed efficiently by incubation with hydroxylamine, a step that should always be performed after labeling of proteins with the method presented here.

Applications and Limitations. In the case of native chemical ligation, the success of the reaction is largely independent of the amino acid residues neighboring the thioester moiety (Dawson and Kent, 2000). The preparation of thioesters using the activation of thiols with trimethylaluminum has been shown to work very efficiently with a wide variety of starting esters (Hatch and Weinreb, 1977). This indicates that the protocol described here will be applicable to succinimidyl esters of other fluorescent dyes and labels without major modifications. Here we focused on Cy5 because of its widespread use and its outstanding fluorescence properties.

The most important feature of the chemistry of native chemical ligation is that conjugation occurs at a unique N-terminal Cys residue. As shown above, it does not matter how many additional internal Cys residues are present in the polypeptide, and no protecting groups are necessary for any of the side-chain functional groups normally found in proteins, similar to what has been found for peptide−peptide ligation (Hackeng et al., 1997). This makes it an ideal approach for attaching a single label even to a large, recombinantly expressed protein containing many cysteine and lysine residues, a situation in which the usual labeling methods do not provide sufficient specificity.

The recombinant expression of proteins with an N-terminal cysteine residue is easily possible either by proteolytic cleavage next to a cysteine residue (Erlanson et al., 1996) or using an intein-based approach (Perler et al., 1997). The more straightforward method is usually the former. It involves constructing an expression vector in which a DNA sequence encoding a recognition site for a highly specific protease like FactorXa followed by cysteine is inserted between an upstream affinity-purification handle (e.g., maltose binding protein MBP, glutathione-S-transferase GST, or a polyhistidine tag) and the appropriate gene fragment of interest. Following

expression and affinity purification, the fusion protein is treated with the protease to give the desired Cys-polypeptide, which can be used directly in the labeling reaction described here and then purified, for instance by HPLC or size-exclusion chromatography.

One potentially limiting property of the method is that it requires refolding of the labeled protein to its native three-dimensional structure, because the reaction does not proceed efficiently in the absence of GdmCl, even for a highly soluble and unstructured peptide like the one used in this study (unpublished observation). The same requirement applies to proteins synthesized by native chemical ligation (Dawson and Kent, 2000) and to many recombinant proteins that aggregate and form inclusion bodies when expressed to high concentrations. For single domain proteins, refolding in vitro is usually simple and proceeds to very high yields, but even most larger multidomain proteins can be refolded very efficiently (Rudolph et al., 1997). Assuming that the N-terminus of the protein is surface-exposed, a serious effect of the additional cysteine or the attached dye on the folding of the protein is improbable, such that the refoldability of the protein under investigation can be investigated before any changes to the sequence are made.

An additional advantage of the dye conjugation to N-terminal cysteins using thioester derivatives is the high stability of the resulting chemical bond compared to many other labeling chemistries. As for the reaction of the commonly used succinimidyl esters with amines, an amide bond is formed, which allows storage and repeated use over an extended period of time. This is an important factor in many of the applications using bioconjugates. The conjugates are also stable in the presence of reducing agents such as dithiothreitol (unpublished observation), which can be important in the investigation of proteins or peptides with reduced cysteine residues.

In summary, the reaction of Cy5-benzyl thioester with N-terminal cysteine residues provides a reliable and uniquely specific method to label polypeptides. The synthesis of thioesters from the respective succinimidyl esters, which are available for most commonly used probes, is a simple and quantitative reaction. The protocol presented here is expected to be applicable to a wide range of labels and polypeptides, including large recombinantly expressed proteins. This will be an important tool for studies requiring the exact positioning of a label in a polypeptide containing multiple sulfhydryl or amino groups.

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