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A Highly Efficient Method for Site-Specific Modification of Unprotected Peptides after Chemical Synthesis

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Abstract: We have developed a highly efficient method for the site-specific attachment of biophysical probes to unprotected peptides after chemical synthesis. This methodology takes advantage of the selective reactivity of an *N*-methylaminoxy amino acid that is appropriately protected for direct incorporation during solid-phase peptide synthesis. The functional *N*-methylaminoxy group is unmasked using normal peptide cleavage conditions and is capable of selective reaction with activated *N*-hydroxysuccinimide esters in the presence of cysteine, lysine and the amino terminus, as demonstrated in model peptides and test proteins. Selective labeling can be accomplished after synthesis using commercially available or chemically sensitive probes. This technology is compatible with the synthesis of C^α-thioester-containing peptides and amide-forming ligations, required steps for the synthesis of proteins by either total chemical synthesis or expressed protein ligation. The *N*-methylaminoxy amino acid can be introduced into different sites by parallel peptide synthesis to generate a polypeptide analogue family with each member possessing a single specifically labeled site. This enables the synthesis of optimized biosensors through combinatorial screening of different attachment sites for maximal response and minimal perturbation of biological activity.

Introduction

Modified peptides and proteins are valuable biophysical tools for studying biological processes, both *in vitro* and *in vivo*.^{1–5} In particular, quantitative live cell imaging of fluorescent proteins and peptides is revolutionizing the study of cell biology.

An exciting recent development within this field has been the construction of peptide and protein biosensors exhibiting altered fluorescence properties in response to changes in their structure or ligand binding.^{2–5} Appropriately labeled fluorescent biomolecules allow spatial and temporal detection of biochemical reactions inside living cells.^{4,5}

The major obstacles in the development of fluorescent biosensors have been: (1) The difficulty in site-specific placement of the dye in the polypeptide and (2) determining exactly which site is optimal for dye placement.¹ Solvent-sensitive dyes must be placed precisely for optimal response to changes in protein structure without interference with biological activity. Also, the need for site-specific incorporation of two dyes without impairment of biological activity has proven a serious limitation for utilization of FRET within a single protein. A potential solution to these problems can be found in a recently developed

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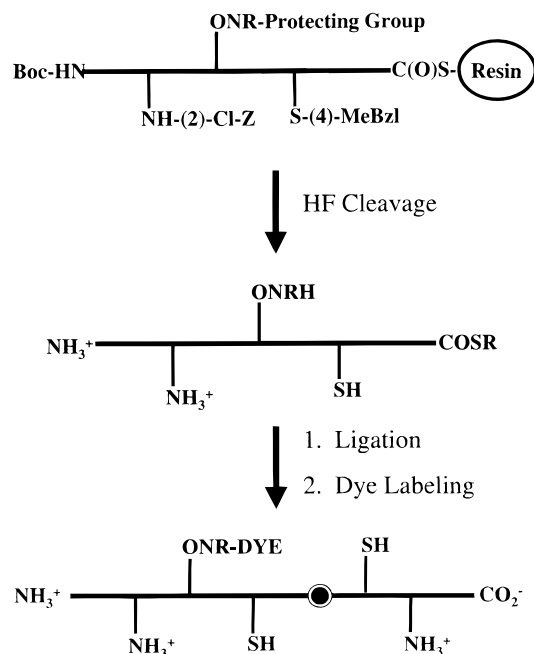


Figure 1. General strategy for site-specific labeling of polypeptides. (Top) The protected aminoxy group is incorporated during solid-phase peptide synthesis (synthesis on thioester-linker resin is shown). (Middle) Cleavage from the resin generates a peptide possessing unprotected side chains, an aminoxy group and a C-terminal thioester. (Bottom) Ligation and subsequent site-specific labeling produces the full-length polypeptide with a single dye attached at the aminoxy nitrogen. R = H or CH₃.

technology, total chemical synthesis of proteins.^{6–9} However, many biophysical probes suitable for fluorescent biosensors or other purposes are not stable to the various conditions used for peptide synthesis, and site-specific incorporation after synthesis is difficult to achieve. The incorporation of an appropriately protected unnatural amino acid during solid-phase peptide synthesis, which can be selectively modified postsynthetically, would resolve these problems.

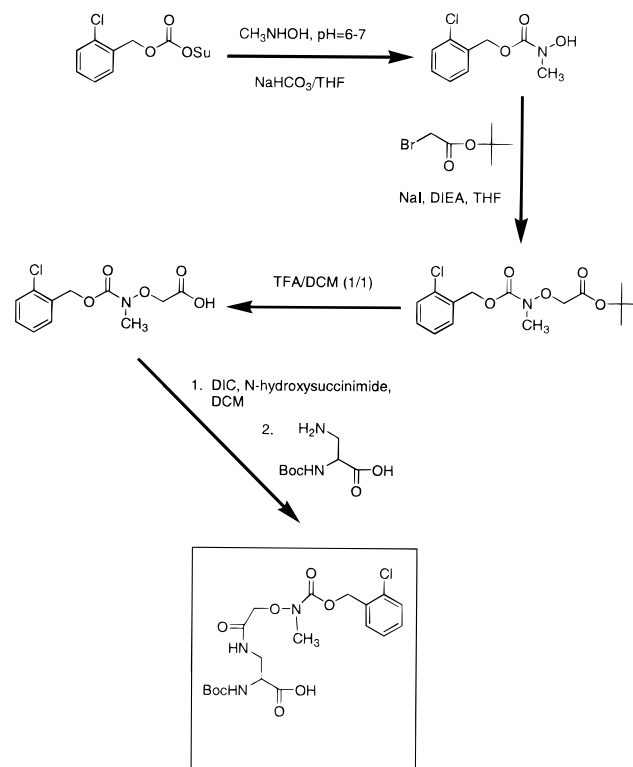
We report here a simple and efficient methodology for site-specific labeling of peptides after synthesis that satisfies the requirements of high yield, selectivity, and compatibility with both solid-phase peptide synthesis and C^α-thioester peptides. The applicability of this method has been tested using model peptides and control proteins.

Results and Discussion

Site-Specific Labeling of the Secondary Aminoxy Group.

Under controlled pH conditions, the low pK_a and enhanced nucleophilicity of an aminoxy group relative to those of other nucleophilic side chains found in peptides suggested the possibility of site-specific reaction with standard electrophiles such as succinimidyl esters (Figure 1).^{10,11} While selective labeling of a primary aminoxy group in the context of an unprotected

Scheme 1. Synthesis of *N*-(2-Cl-Benzyloxycarbonyl)-*N*-methylaminoxy Acetic Acid (**3**) and *N*-(2-Cl-Benzyloxycarbonyl)-*N*-methylaminoxyacetyl-*N*^α-Boc- α,β -diaminopropionic Acid [(SA)Dapa-OH] (**4**)^a



^a *N*-(2-chlorobenzoyloxycarbonyl)succinimide was reacted with excess *N*-methyl hydroxylamine at pH 6–7 to generate the 2-Cl-Z hydroxamic acid (**1**). Compound **1** was reacted with diisopropylethylamine and bromoacetate *tert*-butyl ester to produce *N*-(2-Cl-benzyloxycarbonyl)-*N*-methylaminoxy acetic acid *tert*-butyl ester (**2**), which was directly converted to the free acid (**3**) by treatment with a 1:1 mixture of trifluoroacetic acid and dichloromethane. Compound **3** was used for the synthesis of SA-test peptide and the synthesis of (SA)Dapa-OH (**4**) by condensation with *N*^α-Boc- α,β -diaminopropionic acid. In each case, compound **3** was activated as the *N*-hydroxysuccinimide ester.

peptide was achieved, extensive attempt to utilize the primary aminoxy group during synthesis failed. Even when the primary aminoxy group was protected as the 2-chlorobenzoyloxycarbonyl carbamate, deprotonation allowed rapid acylation, so that it could not be readily incorporated during peptide synthesis. Recently, others have demonstrated a method for deprotection of base labile Fmoc groups in the presence of thioesters:¹² a process which could be utilized to place an aminoxy substituent on the side chain of a lysine within a peptide. However, we alleviated this problem by utilizing a secondary *N*-methylaminoxy group.

We synthesized a test peptide containing both a secondary aminoxy group and nucleophilic amino acids that were most likely to interfere with selective labeling at the aminoxy nitrogen (lysine, cysteine, and the amino terminus). NH₂-AKAARAAA*^aAARACA-CO₂H, here designated SA-test peptide, was synthesized by incorporation and deprotection of *N*-(2-Cl-benzyloxycarbonyl)-*N*-methylaminoxy acetic acid (**3**) during solid-phase peptide synthesis (Scheme 1 and Figure 2). The reactivity of the protected secondary aminoxy group was sufficiently attenuated to remain unreactive during Boc solid-

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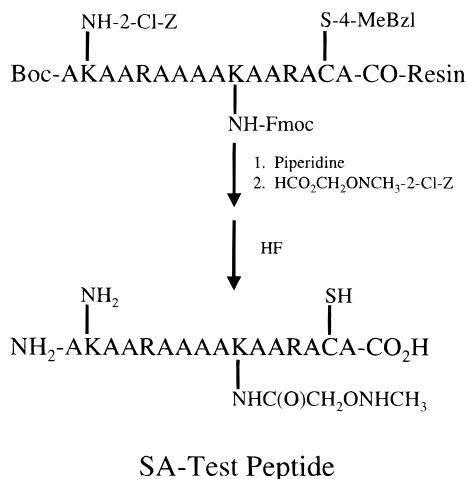


Figure 2. Synthesis of SA-test peptide. For precursor peptide on the resin, the ϵ -amino group of lysine 10 is protected as the Fmoc carbamate. Treatment with piperidine selectively removes the Fmoc-protecting group. Either 2-Cl-Z aminoxy acetic acid or 2-Cl-Z *N*-methylaminoxy acetic acid is then coupled on the lysine side chain to generate, upon standard HF cleavage, PA-test peptide (R=H) or SA-test peptide (R=CH₃) in deprotected form.

Table 1: Labeling of SA-Test Peptide^a

| reaction | buffers | | | dye equiv | percentage SM | percentage | | selectivity |
|-----------|------------------------------------|------|-----------------|-----------|---------------|------------|------|-------------------|
| | gel filtration | TCEP | pH ^b | | | 1Dye | 2Dye | |
| acetate | (NH ₄)HCO ₃ | no | 4.7 | 1.0 | 32 | 56 | 7 | 8:1 |
| acetate | (NH ₄)HCO ₃ | no | 4.7 | 1.5 | 14 | 63 | 13 | 4.5:1 |
| acetate | (NH ₄)HCO ₃ | no | 4.7 | 2.0 | 0 | 78 | 13 | 6:1 |
| acetate | (NH ₄)HCO ₃ | no | 4.7 | 2.4 | 0 | 76 | 17 | 4.5:1 |
| acetate | (NH ₄)HCO ₃ | no | 4.7 | 3.0 | 0 | 71 | 23 | 3:1 |
| citrate | 0.1% TFA | yes | 4.7 | 4.3 | 4.3 | 89.6 | 4.1 | 22:1 |
| carbonate | 0.1% TFA | yes | 9.0 | 0.99 | 13 | 85 | 0 | 51:1 ^c |

^a Conditions and outcome of labeling reactions using SA-test peptide and tetramethylrhodamine *N*-hydroxysuccinimidyl ester. Buffers: acetate = 5% v/v acetic acid buffer (0.9 M) adjusted to pH = 4.7 with NaOH; citrate = 100 mM sodium citrate, pH = 4.7; carbonate = 100 mM sodium bicarbonate, pH = 9.0. All buffers were used in labeling reactions with 50% DMSO. TCEP was used at 5 mM. Equivalents were based on the quantity of SA-test peptide in the reaction. Percentages were determined by HPLC integration: SM = starting SA-test peptide, 1Dye = SA-test peptide with single dye on secondary aminoxy group; 2Dye = doubly labeled SA-test peptide. Selectivity was determined as the ratio of 1Dye/2Dye. ^b pH of the aqueous buffer used in the labeling reaction solvent (50% aqueous buffer, 50% DMSO). ^c Selectivity based on SA-test peptide product with a single dye on the secondary aminoxy nitrogen divided by quantity of SA-test peptide with single dye at alternate sites.

phase peptide synthesis on thioester-linker resins. The 2-Cl-Z protection for the *N*-methylaminoxy amino acid was efficiently removed by standard HF cleavage procedures.

Conditions for selectively labeling the secondary aminoxy group were determined by varying the reaction pH and dye stoichiometry. Labeling with the succinimide ester of tetramethylrhodamine (TMR-OSu) was determined to be optimal in a solvent system consisting of 50% DMSO/50% aqueous acetate buffer at pH of 4.7 with 2 equiv of dye per mol of peptide (Table 1). The crude reaction products were separated from unreacted dye and characterized by RP-HPLC and ESI-MS. Under these conditions, a single molecule of dye was incorporated on the aminoxy group with a 78% yield, based on HPLC quantification. However, side reaction products were also isolated and determined to be either SA-test peptide labeled with two dye molecules (~13%) or acetylated peptide products (~5%). The

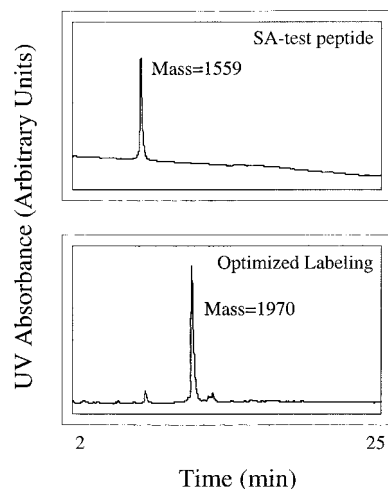


Figure 3. Site-specific labeling and reductive cleavage of SA-test peptide. (Top) HPLC analysis of purified SA-test peptide. (Bottom) HPLC analysis of crude reaction products from optimized labeling conditions. Smaller early eluting peak is unreacted SA-test peptide. Main peak is SA-test peptide with single dye attached to secondary aminoxy nitrogen. Smaller later eluting peaks are SA-test peptide with two dyes attached and an acetylation side-product. Zinc/acetic acid reduction of the singly labeled SA-test peptide yielded a new peak at earlier retention time with a mass corresponding to cleavage of the aminoxy N–O bond. Absorbance was monitored at 214 nm.

selectivity, as defined by the ratio of the peak areas of desired single-labeled product over double-labeled products, was 6/1 (Table 1).

We found that many of the side reactions occurred during size exclusion chromatography in the ammonium bicarbonate solvent used to separate reaction products. Using an acidic solvent system, 0.1% TFA, virtually eliminated multiply labeled side products leading to considerable improvements in both selectivity and yield. Including a mild reducing agent, tris(2-carboxyethyl)phosphine (TCEP), in the reaction buffer also significantly curtailed several minor side reactions revealed by HPLC, especially disulfide formation. Labeling and gel filtration under these optimized conditions produced a 70% recovered yield of labeled SA-test peptide (90% yield based on HPLC quantitation), 90% of which was labeled with only a single dye at the aminoxy amine. The labeling selectivity was increased to 22/1 (Table 1).

The purity of the product was confirmed using RP-HPLC, mass spectroscopy, further chemical reaction, and isolation of purposefully overlabeled products. The labeled peptide eluted as a single peak under all HPLC conditions tested with a mass consistent with that predicted for singly labeled SA-test peptide (mass = 1970). To determine that the labeling site was indeed at the *N*-methylaminoxy group, we utilized a selective zinc/acetic acid reduction procedure to cleave the N–O bond (Figure 3).¹¹ HPLC of the reduction reaction showed >98% conversion of the starting material and a new earlier eluting peak. The mass of this peak (1530 amu) corresponded to the predicted mass of the unlabeled SA-test peptide cleaved at the aminoxy N–O bond. The residual zinc was washed several times with a saturated solution of EDTA in water, which demonstrated that the reduction reaction was complete.

To eliminate the unlikely possibility that the HPLC peak containing isolated single-labeled SA-test peptide product was a mixture of two labeled species, SA-test peptide was reacted under higher pH conditions (pH 9.0) to label all reactive sites.

SA-test peptide contained three nucleophilic labeling sites which would be irreversibly labeled: aminoxy, lysine, and N-terminal amine. Dye labeling at high pH generated a mixture of peptides labeled at all possible combinations of sites with one, two, or three dyes. HPLC analysis of this reaction mixture showed eight peaks, indicated by ESI-MS to correspond to unreacted SA-test peptide, SA-test peptide single-labeled at the aminoxy nitrogen, and six additional peaks corresponding to two single-labeled peptides, three peptides bearing two dyes, and a single triply labeled peptide species. This experiment revealed the HPLC retention times of all of these products, none of which coeluted with the peak identified as SA-test peptide labeled with a single dye on the secondary aminoxy nitrogen.

Site-specific labeling of the aminoxy group could even be achieved at basic pH (Table 1). Using pH 9.0 carbonate buffer in our solvent system, addition of 0.5 equiv of dye produced, after 3 h, ~50% conversion of the starting SA-test peptide to a single peak with the elution time of the desired singly labeled product. After addition of another 0.5 equiv of TMR-OSu and an additional 3 h of reaction time, HPLC showed ~85% conversion to a peak with the retention time of the desired product. Two minor peaks (~2–3% of total peak area), were also apparent and corresponded to the two other single-labeled SA-test peptide species identified in the multiple labeling experiment above. The *N*-hydroxysuccinimide ester of rhodamine clearly showed selective reactivity with the aminoxy group.

The selectivity observed at higher pH cannot be explained by the nucleophilicity of the aminoxy group alone. In fact, others have shown that, in an uncatalyzed reaction with phenyl acetate at high pH, amines are more reactive than O-alkyl aminoxy groups.¹⁰ Therefore, we suggest that kinetic factors are contributing to the selective reactivity of the *N*-methylaminoxy group, even when competing groups are not protonated. Possible reasons for this include: (1) the aminoxy oxygen localizes the nitrogen near the activated ester via formation of a hydrogen bonded "bridged" intermediate, or (2) a base-catalyzed reaction pathway under the conditions of our reaction.¹⁰ This exceptional reactivity has important practical implications, as it can allow the selective labeling of acid-labile polypeptides and synthetic proteins under physiological or basic conditions.

The Secondary Aminoxy Group Is Compatible with C^α-Thioesters and Amide-Forming Ligation. Preparation of proteins by total chemical synthesis often requires the ligation of large polypeptides prepared by solid-phase peptide synthesis on thioester-linker resins.^{6,9} The most generally applicable methods available for ligations are native chemical ligation⁷ and expressed protein ligation.^{8,9} These processes utilize the same basic chemistry to join two peptides, one with an N-terminal cysteine and the other with a C-terminal thioester, through a regioselective and site-specific reaction to generate a larger polypeptide. The application of aminoxy-labeling chemistry to the synthesis of large polypeptides and proteins requires compatibility with these solid-phase peptide synthesis and ligation chemistries.

The optimal approach for utilizing aminoxy-labeling chemistry in the chemical synthesis of proteins is direct incorporation of the aminoxy group as part of an amino acid used during standard solid-phase peptide synthesis. For this purpose, we generated a suitably protected *N*-methylaminoxy amino acid, α -Boc- β -[*N*-(2-chlorobenzoyloxycarbonyl)-*N*-methylaminoxy acetyl]- α,β -diaminopropionic acid [Boc-2-Cl-Z-(SA)Dapa-OH] (**4**), as shown in Scheme 1. This amino acid, referred to as SAOD, was incorporated into the peptide sequence LY-(SAOD)-AG-MPAL thioester by synthesis on TAMPAL thioester-linker

resin, as described in Materials and Methods. (MPAL is the C-terminal mercaptopropionyl-leucine group generated by cleavage of a peptide from TAMPAL resin.¹³)

Ligation of the LY-(SAOD)-AG-MPAL thioester peptide with the peptide, CRANK-NH₂, was tested by means of standard procedures employing phosphate buffer with 6 M guanidine hydrochloride at neutral pH in the presence of 2–3% thiophenol by volume.⁷ The ligation proceeded over 24 h and generated the desired ligation product, LY-(SAOD)-AGCRANK-NH₂, at ~85% yield. The major side product was attributable to modification of unligated CRANK-NH₂ peptide under the ligation conditions (mass = 714.5, data not shown) and was not related to the presence of the aminoxy group. There was also a single time-dependent side reaction, which generated a product of 14 mass units lower than the desired. Using high concentrations of reacting peptides and isolating the ligation product after 24 h reduced this side reaction to acceptable levels (<5%).

We also tested the ligation of a peptide containing multiple potentially reactive functional groups, including a hexahistidine tag useful for affinity chromatography. Coupling CEYRIDRVR-LFVVDKLDNIAQVPRVGAA-HHHHHH to LY-(SAOD)-AG-MPAL thioester proceeded to completion in 5 h with minimal side reactions. In both ligation reactions, there was less than 1% LY-(SAOD)-AG-MPAL self-condensation product, indicating that the aminoxy group and thioester do not appreciably react with one another under the ligation conditions. These results demonstrate that the inclusion of an unprotected aminoxy group in the peptide chain is compatible with native chemical ligation.

Labeling of the two ligation products using tetramethylrhodamine succinimide ester proceeded with selectivity similar to that for the SA-test peptide. HPLC integration indicated that the product of the LY-(SAOD)-AG-MPAL ligation with CRANK-NH₂ was labeled with greater than 95% efficiency and with a selectivity of 34:1. Mass spectral analysis and zinc reduction demonstrated labeling at only the aminoxy group. For the longer hexahistidine-containing polypeptide ligation product, selectivity for the aminoxy group was greater than 10:1, but it was difficult to achieve high yields. The histidines could potentially have been affecting yield and selectivity by catalyzing nucleophilic attack on the succinimide ester of the reactive dye. Inclusion of guanidine hydrochloride in the reaction solvent increased the yield to approximately 50%, indicating that folding or poor solubility of the peptide was a factor in preventing access of the reactive dye to the aminoxy group. Selectivity was also improved, presumably because of the availability of the reactive secondary aminoxy group. Single-site labeling at the aminoxy group was proven by mass spectral analysis of trypsin and α -chymotrypsin digests of the labeled polypeptide product.

Specificity of Labeling in Protein Domains Containing Aminoxy Amino Acids. As a control to establish the selectivity of labeling for aminoxy amino acid, we attempted to label native β -Lactoglobulin with tetramethylrhodamine *N*-hydroxysuccinimide ester. Under our conditions, nonspecific labeling of this 162-amino acid (aa) protein containing 15 lysines and 4 cysteines was minimal (<1%) even after 6 h.

Finally, we synthesized the GTPase binding domain of p21 activated kinase (45 aa, 4 Lys, 1 Cys) with a secondary aminoxy amino acid incorporated at the amino-terminus

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(SAOD-PBD). Previous experiments have demonstrated that PBD domains labeled with fluorescent reporter dyes at this terminus could be used as biosensors of GTPase activation.¹⁴ Labeling of PBD using the new methodology would enable the production of sufficient quantities to apply the biosensors in vivo and in pharmaceutical screening applications and would allow incorporation of sensitive dyes enabling applications within living cells.

SAOD-PBD was readily labeled with Alexa-532 *N*-hydroxy-succinimide ester by titration addition of dye at pH 4.7 over 72 h. The labeling efficiency was commensurate with that reported for the longest model peptide (~50% yield by HPLC quantitation) and there was no indication of multiple labeling. In this case, isolation of labeled SAOD-PBD by RP-HPLC proved difficult. Separation to baseline resolution was not achieved, but small quantities of unlabeled PBD in the labeled product do not preclude the use of the labeled material in biosensor applications. Previous reports indicate that separation of labeled product from starting polypeptide is highly dependent on the specific peptide and the attached dye.

In summary, these results clearly demonstrate that the optimized site-specific labeling chemistry reported here is compatible with the steps required for the preparation of proteins by total chemical synthesis.

Conclusions

In this paper, we report a simple and efficient synthetic methodology for site-specific labeling of peptides after synthesis that satisfies the requirements of high yield, selectivity and compatibility with both solid-phase peptide synthesis and C α -thioester peptides. The approach and primary advantages can be summarized as follows: (1) A protected *N*-methylaminoxy amino acid which can be incorporated into peptides by means of optimized solid-phase Boc synthesis procedures¹⁶ has been synthesized. (2) Labeling procedures have been optimized to yield highly efficient and specific modification of the *N*-methylaminoxy nitrogen in the presence of unprotected competing nucleophiles, including cysteine, lysine, and the amino terminus. (3) The electrophile used in labeling, an activated carboxylic ester, is readily available in the majority of commercially available fluorescent dyes and labels.^{17a} (4) Labeling of the *N*-methylaminoxy group occurs after synthesis and purification, thus enabling the use of chemically sensitive fluorophores and labels that would otherwise not survive earlier synthetic procedures. (5) This synthetic methodology is compatible with the critical steps required for the synthesis of proteins by total chemical synthesis or expressed protein ligation, namely synthesis of C α -peptide thioesters and amide-forming ligations.^{6–9} (6) Combinatorial screening of both the dye used and its placement¹⁸ could enable the rapid synthesis of optimally labeled polypeptide-based biosensors.

Other procedures for site-specific modification of polypeptides have been described, including chemically selective labeling in solution¹⁹ and on resin-bound peptides,²⁰ introduction

of ketone amino acids through synthetic procedures^{21–25} or molecular biology,²⁶ and expressed protein ligation.^{8,9} While each method has utility for producing a particular class of biosensor, all have limitations that restrict their general use. Labeling of natural amino acid side chains in solution is often impractical because of the existence of many other competing nucleophiles. To use unnatural amino acids, such as those bearing ketones for selective labeling, requires the synthesis of dye constructs or amino acids that are difficult to make and not available commercially. Peptide labeling on the resin limits usable fluorophores to those that can survive the harsh conditions used in synthesis and cleavage of the labeled polypeptide. The procedure we have developed resolved many of these issues and should be generally applicable to a wide range of polypeptides and biophysical probes.

Materials and Methods

General. For column chromatography, silica gel (230–400 mesh) was used in standard glass columns with gravity or air pressure. Reversed-phase high performance liquid chromatography (RP-HPLC) was performed on a Waters HPLC system with UV detection at 214 nm using either a Vydac C-18 analytical column (5 μ m, 0.46 \times 25 cm), a Waters RCM 8 \times 10 module equipped with a semipreparative Delta Pack C-18 Radial Pack cartridge column (15 μ m, 8 \times 100 mm) from Millipore, or a Vydac C-18 preparative scale column (15 μ m, 1.0 \times 25 cm). Linear gradients of solvent B (0.09% TFA in 90% acetonitrile/10% water) in solvent A (0.1% TFA in water) were used for all HPLC chromatographic separations.^{6b}

Mass spectra of peptides were obtained either with a Sciex API-III electrospray ionization (ESI) triple-quadrupole mass spectrometer (PE Biosystems, Foster City), or matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) instruments from Thermo Bioanalysis (Thermo Bioanalysis, Ltd., UK) or Kratos Analytical (Chestnut Ridge, NY). For ESI-MS, the observed masses reported were derived from the experimental *m/z* states for all observed charge states of a molecular species using the program MacSpec (Sciex, version 2.4.1) for electrospray mass spectrometry. MALDI-MS observed masses were relative to internal calibration using α -cyano-hydroxycinnamic acid or sinipinic acid matrices. Calculated masses reported were derived from either MacProMass (Terry Lee and Sunil Vemuri, Beckman Research Institute, Duarte, CA) or PAWS (Version 8.1.1, ProteoMetrics) and reflect the average isotope composition of the singly charged molecular ion. Proton nuclear magnetic resonance spectrometry was recorded on a Bruker AC-250 mass spectrometer and data were analyzed using WinNMR (Bruker Instruments). Ultraviolet–visible spectroscopy was performed on a Hewlett-Packard photodiode-array spectrophotometer.

Boc-L-amino acids were purchased from Novabiochem (La Jolla, CA) or Bachem Bioscience, Inc. (King of Prussia, PA). [[4-(Hydroxymethyl)phenyl]-acetamido]methyl (–OCH₂–Pam) resin was purchased from PE Biosystems (Foster City, CA), and methylbenzhydrylamine (MBHA) resin was purchased from Peninsula Laboratories, Inc. (San Carlos, CA). Solvents were synthesis grade or better and were purchased from Fisher Scientific (Tustin, CA). Trifluoroacetic acid (TFA) and anhydrous hydrogen fluoride were purchased from Halocarbon (New Jersey) and Matheson Gas (Rancho Cucamonga, CA). Dyes were obtained from Molecular Probes (Eugene, OR). All other reagents were analytical grade or better and were purchased from

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Aldrich (Milwaukee, WI), Lancaster (Windham, NH), Peptides International (Louisville, KY) or Richelieu Biotechnologies (Montreal, Canada).

Peptide Segment Synthesis. Synthesis of peptides was carried out manually using optimized stepwise solid-phase synthesis methods with in situ neutralization and HBTU activation procedures for Boc chemistry on either $-\text{OCH}_2\text{-Pam}$, MBHA, or Trt-protected mercaptopropionyl-Leu (TAMPAL) resin.^{13,16} Standard Boc-protecting group strategies were employed.^{6b,16} Coupling was monitored by quantitative ninhydrin assay after 15 min coupling cycles.²⁷ After chain assembly, standard deprotection and cleavage from the resin support was carried out by treatment at 0 °C for 1 h with anhydrous HF containing either 10% *p*-cresol or anisole as scavenger. Purification was performed using RP-HPLC.^{6b}

Synthesis of TAMPAL Resin.¹³ MBHA resin (2.5 g, 0.865 mmol/g, 2.16 mmol of amine) was swelled in DMF. Boc-Leu-OH (1.1 g, 4.4 mmol) was activated with HBTU (8 mL, 0.5M solution) and DIEA (2 mL) and then coupled to the MBHA resin until complete reaction by ninhydrin assay. The N^α -Boc group of the linked leucine was removed with neat TFA, and then *S*-Trt- β -mercaptopropionic acid (1.5 g, 4.3 mmol), activated in the same manner as Boc-Leu-OH, was added to the deprotected Leu-MBHA resin and allowed to couple until complete reaction. The *S*-Trt- β -mercaptopropionyl-Leu-MBHA resin was washed extensively with DMF and then with DCM/MeOH (1/1) and finally dried in vacuo to yield 3.39 g of thioester resin. Substitution calculated by weight gain yielded 0.549 mmol/gram.

Deprotection of TAMPAL Resin. *S*-trityl protection was removed by two 5 min treatments with 95% TFA/5% triisopropylsilane.¹³ The deprotected resin was extensively washed with DMF before coupling the first amino acid, activated using optimized in situ neutralization protocols.

Synthesis of *N*-(2-Cl-Benzoyloxycarbonyl)-*N*-methylhydroxylamine (1).¹⁰ *N*-methylhydroxylamine hydrochloride (0.95 g, 11.37 mmol) was dissolved in 3 mL of water with rapid stirring. The pH of this solution was adjusted to 6–7 by dropwise addition of a saturated solution of sodium bicarbonate. 2-Chlorobenzoyloxycarbonyl-*N*-hydroxysuccinimidyl carbonate (1.2 g, 4.23 mmol) was dissolved in 4 mL of THF and added slowly to the rapidly stirring solution of neutralized *N*-methylhydroxylamine. After stirring at room temperature for 14 h, the reaction was quenched with 20 mL of saturated sodium bicarbonate and extracted three times with 20 mL of ethyl acetate. The combined ethyl acetate layers were washed once with saturated sodium bicarbonate and dried over anhydrous sodium sulfate, and the solvent was removed in vacuo to yield 0.77 g (3.77 mmol, 84%) of an off-white solid. TLC $R_f = 0.2$ (Hex/EtOAc/AcOH 80/20/1). ¹H NMR: 3.23(s, 3H), 5.25 (s, 2H), 7.24 (m, 2H), 7.37 (m, 2H). HRMS: Expected = 216.0427, Observed = 216.0425.

Synthesis of *N*-(2-Cl-Benzoyloxycarbonyl)-*N*-methylaminoxy Acetic Acid-*tert*-butyl ester (2).²⁸ Compound 1 (0.96 g, 4.71 mmol) was dissolved at room temperature in 10 mL of THF with rapid stirring. To the solution was added bromoacetate *tert*-butyl ester (1.05 g, 5.38 mmol) and then sodium iodide (1.5 g, 10.01 mmol) followed by DIEA (2.5 mL, 15.92 mmol). The reaction changed to an orange-yellow color after addition of sodium iodide. The reaction was quenched with 30 mL of water after complete reaction (~3 h) and extracted three times with ethyl acetate. The combined ethyl acetate layers were dried over sodium sulfate, and the ethyl acetate was removed in vacuo. The resultant oily solid was purified by silica chromatography on 230–400 mesh silica gel using hexanes/ethyl acetate/acetic acid (80/20/1) to yield 1.40 g (4.29 mmol, 90%) of a pure yellow oil. TLC $R_f = 0.5$ (Hex/EtOAc/AcOH 80/20/1). ¹H NMR: 1.46 (s, 9H), 3.29 (s, 3H), 4.36 (s, 2H), 5.26 (s, 2H), 7.24 (m, 2H), 7.40 (m, 2H). HRMS: Expected Mass = 330.1108, Observed Mass = 330.1104.

Synthesis of *N*-(2-Cl-Benzoyloxycarbonyl)-*N*-methylaminoxy Ace-

tic Acid (3).²⁹ Compound 2 (1.1 g, 3.30 mmol) was dissolved in 4 mL of DCM, and with rapid stirring, neat TFA (5 mL) was added dropwise over 2 min at room temperature. After 1 h, the reaction was quenched with 20 mL of water and extracted three times with DCM, and then the combined DCM layers were dried over sodium sulfate. The DCM was removed in vacuo to yield 0.9 g (3.28 mmol, 99%) of an off-white solid. TLC $R_f = 0.2$ (Hex/EtOAc/AcOH 80/20/1). ¹H NMR: 3.23 (s, 3H), 4.50 (s, 2H), 5.32 (s, 2H), 7.28 (m, 2H), 7.40 (m, 2H). HRMS: Expected Mass = 274.0482, Observed Mass = 274.0479.

Synthesis of *N*-(2-Cl-Benzoyloxycarbonyl)-*N*-methylaminoxy-acetyl- α -Boc- α,β -Diaminopropionic Acid [(SA)Dapa-OH](4).^{25,30} *N*-(2-Chlorobenzoyloxycarbonyl)-*N*-methylaminoxy acetic acid (3) (2.5 g, 9.2 mmol) was activated with *N*-hydroxysuccinimide (2.11 g, 2 equiv) and DIC (1.440 mL, 1.0 equiv) in 20 mL of DCM. This reaction was rapidly stirred at room temperature for 2 h prior to the addition of N^α -Boc- α,β -diaminopropionic acid (2.3 g, 1.2 equiv) and DIEA (3.20 mL, 2 equiv). After 4 h, the DCM solvent was removed in vacuo, and 50 mL of ethyl acetate was added. The ethyl acetate layer was washed twice with 0.5 M acetate buffer, pH = 4.0 and then twice with 0.1 N sulfuric acid. The combined acid washes were then washed with 50 mL of ethyl acetate. The combined ethyl acetate layers were dried over sodium sulfate and then concentrated in vacuo to yield a viscous yellow oily solid. This solid was subjected to three hexane precipitations from diethyl ether to yield 2.16 g (51% yield) of an off-white solid. TLC $R_f = 0.2\text{--}0.4$ (Hex/EtOAc/AcOH 30/70/0.5). ¹H NMR: 1.45 (s, 9H), 3.16 (s, 3H), 3.54 (d-of-t, 1H, $J = 14.3, 4.6$ Hz), 3.93 (m, 1H, $J = 14.3, 7.5, 4.6$ Hz), 4.31 (s, 0.5H), 4.38 (s, 1H), 4.45 (s, 1H), 4.51 (s, 0.5H), 5.34 (s, 2H), 5.97 (broad-d, 1H, $J = 7.3$ Hz), 7.30 (m, 2H), 7.43 (m, 2H), 8.50 (broad-s, 1H). HRMS: Expected Mass = 460.1487, Observed Mass = 460.1480.

Synthesis of Secondary Aminoxy Test Peptide (SA-test peptide). The SA-test peptide, $\text{NH}_2\text{-AKAARAAA}*AARACA\text{-CO}_2\text{H}$, was synthesized with Lys 10 side chain Fmoc protection as described previously.³¹ Incorporation of the secondary aminoxy group was accomplished by coupling 2-Cl-Z-protected *N*-methylaminoxyacetic (300 mgs, 1.09 mmol) activated with diisopropylcarbodiimide (157 μL , 1.00 mmol) and *N*-hydroxysuccinimide (140 mg, 1.22 mmol) in 2 mL of DCM for 1–2 h and then diluted with 2 mL of DMF just prior to coupling to the ϵ -amino group of Lys 10. Optimized coupling, cleavage, and purification protocols were utilized.^{6b,10} Amino acid analysis was consistent with the desired peptide. Expected Mass = 1560, Observed Mass = 1559.

Synthesis of LY-(SAOD)-AG-MPAL-Thioester. LY-(SAOD)-AG-MPAL-thioester was synthesized using optimized in situ neutralization protocols for Boc chemistry on TAMPAL resin.^{13,16} Coupling of the N^α -Boc-(SA)Dapa-OH amino acid was accomplished by reacting the in situ activated *N*-hydroxysuccinimide ester to the deprotected amino-terminal nitrogen of alanine.³¹ (SA)Dapa-OH (4) (230 mgs, 0.5 mmol) was dissolved in 1 mL of DCM and *N*-hydroxysuccinimide (115.1 mgs, 1.0 mmol) and DIC (74.4 μL , 0.47 mmol) were added. The reaction was mixed briefly and allowed to activate for 1–2 h at room temperature prior to coupling to the deprotected N-terminus of the peptide chain. After this coupling, no further modifications of the synthetic protocols were required. Expected mass = 797, Observed mass = 797.

Ligation of LY-(SAOD)-AG-MPAL-Thioester with CRANK-NH₂ Peptide. LY-(SAOD)-AG-MPAL-thioester (3 mg, 3.8 μmol) was dissolved in 100 μL of 50 mM phosphate buffer containing 6 M guanidine hydrochloride, pH = 7.2. To this solution was added CRANK-NH₂ peptide dissolved in 100 μL of the same phosphate buffer and 3 μL of thiophenol.⁷ The reaction was monitored by analytical reversed-phase HPLC. After 24 h, the ligated product, LY-(SAOD)-AGCRANK-NH₂, was isolated by semi-preparative reversed-phase HPLC (gradient = 10–50% B over 60 min) and lyophilized to yield

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a fluffy white solid. Amino acid analysis was consistent with the desired product peptide. Expected Mass = 1168, Observed Mass = 1168.

Ligation of LY-(SAOD)-AG-MPAL-Thioester with CEYRIDRVR-LFVDKLDNIAQ-VPRVGAA-HHHHHH. LY-(SAOD)-AG-MPAL (0.3 mg, 0.37 mmol) and CEYRIDR-VRLPFVDKLDNIAQ-VPRVGAA-HHHHHH (1.5 mg, 3.8 mmol) were subjected to the same ligation and purification conditions as those described above to yield 1.0 mg (58% yield) of a white fluffy solid. Expected Mass = 4518, Observed Mass = 4517.

Synthesis of Amino-Terminal P21 Binding Domain (PBD) Peptide Fragment, (SAOD)-KKKEKERPEISLPSDFEHTIHVGFDA-MPAL Thioester. The amino-terminal PBD thioester containing a secondary aminoxy group was synthesized as described above using TAMPAL resin. HF cleavage utilizing *p*-cresol scavenger followed by HPLC purification yielded (SAOD)-KKKEKERPEISLPSDFEHTIHVGFDA-MPAL containing two DNP groups protecting the histidines. Mass Expected = 3745, Mass Observed = 3745.

Synthesis of Carboxy-Terminal of P21 Binding Domain (PBD) Peptide Fragment, CTGEFTGMPEQWARLLQT. The native carboxy-terminal half of PBD was synthesized using standard Fmoc synthesis protocols by the Scripps Peptide and Protein Core Facility. Mass Expected = 2068, Mass Observed = 2068.

Synthesis of SAOD-Modified PBD, SAOD-KKKEKERPEISLPSDFEHTIHVGFDA-CTGEFTGMPEQWARLLQT. SAOD-KKKEKERPEISLPSDFEHTIHVGFDA-MPAL (1.5 mg, 0.4 mmol) was ligated to 1 mg (4.8 mmol) of carboxy-terminal fragment, CTGEFTGMPEQWARLLQT, as described above. After 48 h, the ligated PBD proteins were isolated by RP-HPLC and lyophilized, 1.5 mg (70% yield), Mass Expected = 5262, Mass Observed = 5262.

Selective Labeling of SA-Test Peptide with Tetramethylrhodamine *N*-Hydroxysuccinimidyl ester. A solution of SA-test peptide (3.396 $\mu\text{g}/\mu\text{L}$, 2.18 mM) in 5% acetate buffer, pH = 4.7 incorporating 5 mM TCEP was utilized for labeling. A stock solution of dye (5 $\mu\text{g}/\mu\text{L}$, 9.5 mM) was made by dissolving TMR-OSu in neat DMSO. For each reaction, the dye stock was diluted so that the desired number of dye equivalents could be added in 20 μL of DMSO. The following equivalents of dye were tested: 1.2, 1.5, 1.8, 2.0, 2.4, 3.0, and 4.3. With constant stirring, 20 μL of dye solution was added in two 10 μL aliquots to 20 μL of peptide solution at room temperature. The second aliquot of dye in DMSO was added 10 min after the initial dye addition. After complete addition of dye, the reaction was briefly vortexed and then incubated at room temperature. After 3 h, the labeled reaction product(s) were separated from unreacted dye by gel filtration on Sephadex G-10 or G-15 columns using either 100 μM ammonium bicarbonate or, after optimization, 0.1% TFA in water. The individual peptide product(s) were then separated by RP-HPLC and analyzed by ESI-MS. Mass Expected = 1971, Mass Observed = 1970.

Non-Selective Labeling of SA-Test Peptide with Tetramethylrhodamine *N*-Hydroxysuccinimide ester. SA-test peptide (3.396 $\mu\text{g}/\mu\text{L}$, 2.18 mM) was dissolved 100 mM sodium carbonate, pH = 9.01 containing 5 mM TCEP. 18 μL of neat DMSO was added to 20 μL of peptide solution. With rapid mixing, 1 μL of stock dye solution in DMSO was added to this mixture. Upon completion of the addition, the reaction was vortexed briefly and then incubated at room temperature. After 3 h, a 15 μL aliquot was removed and evaluated by RP-HPLC and ESI-MS. This process was repeated until significant levels of reaction were detected by the formation of single-labeled SA-lysine test peptide products.

Selective Labeling of LY-(SAOD)-AGCRANK-NH₂ and LY-(SAOD)-AGCEYRIDRVR-LFVDKLDNIAQVPRVGAA-HHHHHH with Tetramethylrhodamine *N*-Hydroxy-succinimidyl Ester. A

sample of 20 μL of LY-(SAOD)-AGCRANK-NH₂ (2.5 $\mu\text{g}/\mu\text{L}$, 2.18 mM) in 200 mM citrate buffer, pH = 4.7, 5 mM TCEP was labeled using 4.3 equiv of dye in DMSO, purified, and analyzed. Expected Mass = 1580, Observed Mass = 1580. A 20 μL sample of LY-(SAOD)-AGCEYRIDRVR-LFVDKLDNIAQVPRVGAA-HHHHHH (9.7 $\mu\text{g}/\mu\text{L}$, 2.12 mM) in 200 mM citrate buffer, pH = 4.7, containing 5 mM TCEP and 3 M guanidine hydrochloride was labeled using a modified procedure. Eighteen microliters of a solution of tetramethylrhodamine *N*-hydroxysuccinimide (10 $\mu\text{g}/\mu\text{L}$) in DMSO was added in 6 μL aliquots over 15 min with rapid mixing. The reaction was incubated at room temperature for 5 h prior to gel filtration/RP-HPLC purification and mass spectral analysis. Expected Mass = 4930, Observed Mass = 4929.

Labeling of β -Lactoglobulin with Tetramethylrhodamine *N*-Hydroxysuccinimide Ester. A solution of tetramethylrhodamine *N*-hydroxysuccinimide ester (10 μL , 10 mg/mL) in DMSO (9.5 equiv compared to protein), was added to a solution containing 10 μL of DMSO and 20 μL of a solution of β -lactoglobulin (20.3 mg/mL or 1.1 mM) in 2.8 M guanidine hydrochloride with 5 mM TCEP (pH 4.7). After 3 h, protein was separated from unreacted dye by gel filtration, and labeling was determined by analysis of the dye-to-protein ratio (protein concentration was determined by the method of Waddell and ϵ for tetramethylrhodamine in phosphate buffer, pH = 8.0 of 81 000).¹⁷

Labeling of PBD Proteins with Alexa-532 *N*-Hydroxysuccinimide Ester. SAOD-modified PBD protein (150 μg) was dissolved in 105 μL of 200 mM sodium citrate buffer, pH = 4.8, containing 5 mM TCEP (protein concentration \sim 0.28 mM). A solution of Alexa-532-OSu in DMSO (dye concentration \sim 10 mg/mL in DMSO) was titrated into the protein solution in 5 μL aliquots over 72 h. Four hours after each addition, the extent of labeling was determined by RP-HPLC and MS. Labeling was continued until quantities of double-labeled PBD were obtained (SAOD-modified PBD). Alexa-532 labeled SAOD-modified PBD, Mass Expected = 5871, Mass Observed = 5870.

Zinc/Acetic Acid Reduction of the *N*-Methylaminoxy *N*-O Bond in Peptides. Reductive cleavage of the *N*-O bond was performed using zinc and aqueous acetic acid.¹¹ Effervescence in the reaction was evident after a few seconds and subsided after 60–120 min. After 14 h, the reaction supernatant was analyzed by RP-HPLC and ESI-MS. Reduction of labeled SA-Test peptide, Expected Mass = 1530, Observed Mass = 1530: Reduction of labeled (SAOD)-AGCRANK-NH₂: Expected Mass = 1139, Observed Mass = 1139.

Trypsin/Chymotrypsin Cleavage of Labeled LY-(SAOD)-AGCEYRIDRVR-LFVDKLDN-IAQVPRVGAA-HHHHHH Peptide. A solution of either trypsin or α -chymotrypsin (10 μL , 0.05 mg/mL) in 25 mM ammonium carbonate (without pH adjustment) was added to 5 μL of a 10–20 $\mu\text{g}/\mu\text{L}$ solution of pure tetramethylrhodamine-labeled peptide in water (final concentration of protease is 0.033 mg/mL). The reaction was incubated at room temperature for 24 h prior to analysis of peptide fragments by MALDI-MS.

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