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Cleavable Biotin Probes for Labeling of Biomolecules via the Azide – Alkyne Cycloaddition

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Abstract

The azide-alkyne cycloaddition provides a powerful tool for bio-orthogonal labeling of proteins, nucleic acids, glycans, and lipids. In some labeling experiments, e.g., in proteomic studies involving affinity purification and mass spectrometry, it is convenient to use cleavable probes that allow release of labeled biomolecules under mild conditions. Five cleavable biotin probes are described for use in labeling of proteins and other biomolecules via the azide – alkyne cycloaddition. Subsequent to conjugation with metabolically labeled protein, these probes are subject to cleavage with either 50 mM $Na_2S_2O_4$, 2% $HOCH_2CH_2SH$, 10% HCO_2H, 95% CF_3CO_2H, or irradiation at 365 nm. Most strikingly, a probe constructed around a dialkoxydiphenylsilane (DADPS) linker was found to be cleaved efficiently when treated with 10% HCO_2H for 0.5 h. A model GFP protein was used to demonstrate that the DADPS probe undergoes highly selective conjugation and leaves a small (143 Da) mass tag on the labeled protein after cleavage. These features make the DADPS probe especially attractive for use in biomolecular labeling and proteomic studies.

Introduction

The azide-alkyne cycloaddition is selective, efficient, and broad in scope – a paradigmatic example of a ‘click’ reaction.[1] The discovery that copper catalysis allows the cycloaddition to be effected under mild conditions[2-3] has stimulated numerous studies of labeling of proteins,[4-5] glycans,[6-7] lipids[8-9] and RNA.[10] For example, we recently reported the Bio-orthogonal Non-Canonical Amino Acid Tagging[4] (BONCAT) method for selective identification of newly synthesized proteins in cells. In the BONCAT approach, newly synthesized proteins are distinguished from the pre-existing pool of proteins by co-translational incorporation of a reactive non-canonical amino acid (e.g., L-homopropargylglycine (**1**), Figure 1). Azide or alkyne functional groups on the side chains of such non-canonical amino acids[11,12] can serve as bio-orthogonal handles for selective

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Supporting Information **Available**. Sequence and mass spectrum of protein **18**; ^{1}H NMR spectra for compounds **4**, **5**, **6**, **9**, **11**, **12**, **14a** and **14b**; ^{13}C NMR spectra for compounds **4**, **5**, **6**, **9**, **12**, **14a** and **14b**; and MS/MS spectra of tryptic fragments are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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conjugation to affinity probes through the copper-catalyzed azide-alkyne cycloaddition reaction.[13] Once conjugated, newly synthesized proteins can be selectively enriched through conventional affinity purification protocols that exploit the biotin-streptavidin interaction. Proteins are released from the resin and identified by mass spectrometry. Azide or alkyne functional groups incorporated into proteins can also be used for cell-selective labeling of newly synthesized proteins.[14,15]

Avidin-based isolation of biotin-labeled proteins has found widespread use; however, the strength of biotin-avidin interaction ($K_d \sim 10^{-15}$ mol/L) can make recovery of proteins from affinity resins challenging. Conventional methods to elute biotinylated proteins from immobilized avidin include: (i) denaturation of streptavidin by boiling the resin in a denaturing buffer[16] that may include high concentrations of chaotropic salts, (ii) trypsin digestion of proteins while they are bound to the resin,[17] or (iii) elution of proteins with excess free biotin.[18] These protocols can co-elute contaminant proteins by releasing non-specifically bound proteins and/or naturally biotinylated proteins concurrently with labeled proteins.[19] In addition, some of these methods can cause elution of high levels of resin-based peptides along with the proteins of interest, resulting in further sample contamination. For these reasons, additional purification techniques can be required prior to analysis by mass spectrometry. Furthermore, most biotin probes require "spacers" to ensure accessibility of the biotin to the avidin on the resin,[20] thus these probes can be quite large molecules. Ideally, any modification of the proteins of interest should be small to facilitate protein identification by mass spectrometry. These challenges have prompted the proteomics community to explore the use of cleavable probes.[21–25]

Cleavable biotin probes often consist of five parts: (i) the reactive handle, (ii) a spacer between the reactive handle and the cleavable moiety (iii) the cleavable moiety, (iv) a spacer between the cleavable moiety and biotin and (v) the biotin (Figure 2). In view of the importance of the copper catalyzed azide–alkyne cycloaddition in bioconjugation,[4–10] we designed a set of azide-functional, cleavable biotin probes, and we sought to compare the performance of different cleavable moieties embedded between the azide group and the biotin. An ideal probe would leave behind a small modification on the protein that would not complicate mass spectrometry; the spacer between the azide and the cleavable moiety should be designed accordingly. The probe should be cleavable under conditions that are mild enough to avoid release of streptavidin or of proteins non-specifically bound to the resin, and that do not modify the proteins in any way. Cleavage must also allow rapid, efficient protein recovery in a manner that is compatible with downstream analytical procedures.

Several cleavable biotin probes have been reported previously, including the commercially available acid-cleavable probe used in the Cleavable Isotope Coded Affinity Tag (cICAT) method.[21] Other probes have used disulfides,[22] diazobenzenes that are cleavable under reducing conditions,[15, 23] or photocleavable linkers.[24] In order to design the most effective probe for use in bioconjugation via the azide–alkyne cycloaddition, we explored all of these cleavage approaches. In addition, we were tempted by the acid- and fluoride-sensitive silicon-oxygen bond found in a variety of protecting groups used in organic synthesis[26] as well as in the cleavable motifs used in solid phase synthesis.[27] Five cleavable biotin probes of the general structure shown in Figure 2, all bearing an azide group as the reactive site for bioconjugation, were prepared. Probe performance was evaluated by examining conjugation to and release from a test protein (GFP) bearing a single copy of the non-canonical amino acid **1**. One probe, designed around the acid sensitive dialkoxydiphenylsilane (DADPS) motif, was cleaved under especially mild conditions (10% HCO_2H, 0.5 h). This probe exhibits high labeling selectivity, and leaves a small (143 Da) mass tag on the labeled

protein after cleavage. These features make the DADPS probe especially attractive for use in proteomics and other biomolecular labeling studies.

Results and discussion

Synthesis of Cleavable Probes

In order to prepare a photocleavable biotin probe, the methyl ketone **3**[24] was obtained in three steps from commercially available 5-methyl-2-nitrobenzoic acid (**2**) (Scheme 1). Radical bromination at the benzylic position followed by azide displacement gave compound **4** in 75% overall yield. Reduction of the ketone by $NaBH_4$ and reduction of the azide under Staudinger conditions[28] afforded compound **5**. Amide formation using the commercially available NHS-LC-biotin followed by derivatization of the benzylic alcohol by treatment with disuccinimidyl carbonate and 3-azidopropylamine afforded the desired photocleavable biotin probe (**6**).

We then turned our attention to the diazobenzene motif, which is cleavable via reduction with sodium dithionite ($Na_2S_2O_4$). Compound **8**[23] was prepared in two steps from 4-aminobenzoic acid (**7**, Scheme 2). Amide formation with 3-azidopropylamine introduced the azide handle, and following Fmoc deprotection with piperidine, the liberated amine was coupled with NHS-LC-biotin to afford the desired cleavable biotin probe (**9**).

The acid- and fluoride-sensitivities of the silicon-oxygen bond commonly found in cleavable protecting groups used in organic synthesis can be tuned by varying the extent of steric hindrance around the silicon atom.[26] In designing a cleavable silane probe, we placed two phenyl substituents on silicon, reasoning that smaller groups might render the probe prone to premature cleavage. For the same reason, a tertiary alkoxy group was placed on the biotin side of the silicon atom. The acid stability of the dialkoxydiphenylsilane motif has been reported to increase by two orders of magnitude upon replacement of a methoxy group by a *tert*-butoxy substituent.[29] The biotin NHS ester **10** was treated with 1-amino-2-methyl-propan-2-ol to afford the tertiary alcohol **11** (Scheme 3). The latter was treated first with excess dichlorodiphenylsilane (DCDPS) and then with excess 6-azidohexanol to afford the acid cleavable biotin probe **12** after silica gel chromatography.

The azido disulfide biotin probe **14a** was prepared by treating the commercial NHS ester biotin derivative **13** with 3-azidopropylamine (Scheme 4). An alkynyl disulfide probe (**14b**) was prepared by treatment of **13** with propargylamine. Acid-cleavable probe **16** was prepared by treating the commercially available iodoacetamide biotin derivative **15** of undisclosed structure with 3-azidopropanethiol (Scheme 5).

Evaluation of Cleavable Probes

With the biotin probes in hand, we proceeded to assess the labeling and cleaving efficiencies of each probe with a model protein. A His-tagged green fluorescent protein (GFP) **17** was engineered to contain a single methionine (Met) site, at the initiator position (Scheme 6); replacement of Met by **1** generated the alkyne-labeled protein **18.** Use of a model protein bearing a single alkyne site reduced complications that might arise from multiple labeling and cleaving events. The model protein was obtained by established protocols for *in vivo* synthesis of proteins containing non-canonical amino acids.[17] Briefly, a Met-auxotrophic strain of *Escherichia coli* harboring a plasmid coding for IPTG-inducible expression of the model protein was grown in M9 medium supplemented with all twenty canonical amino acids and ampicillin. Upon reaching a cell density corresponding to OD_{600}=1.0, cells were collected by centrifugation and washed twice in chilled 0.9% NaCl solution. Following re-suspension in M9 medium supplemented with 1 mM **1** and all twenty canonical amino acids except Met, protein synthesis was induced with IPTG. Following expression, His-tagged

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GFP was isolated by nickel-affinity chromatography under native conditions. Mass spectrometric analysis of protein **18** showed a single peak at 28092 Da, indicating near-quantitative replacement of Met by **1**; no signal was detected at 28114 Da, the mass of the Met form of the protein (see supporting information).

Biotinylated proteins **19a-e** were obtained by treatment of **18** with each of the biotin probes (**6**, **9**, **12**, **14a**, and **16**). The Met form of the protein (**17**) was also treated under the same reaction conditions with every probe as a selectivity control. Western blot analysis with fluorescent anti-His antibody was used as a measure of the total protein content of each sample; fluorescent streptavidin was used to visualize biotinylated protein. Comparison of the streptavidin signals obtained from the control protein **17** with those from proteins **19a-e** allowed quantitative comparison of the selectivity of the probes (Figure 3 and Table 1). Probe **14a** was significantly less selective than the others (see figure 3, lane 10b), likely due to disulfide exchange with one of the three cysteine residues on the model protein. The relative selectivity of probe **14a** was therefore arbitrarily designated as unity (see Table 1). Probes **6**, **9** and **16** were of comparable selectivity, showing roughly ten-fold less non-specific labeling than probe **14a**. Silane probe **12** was remarkably more selective (see Table I), exhibiting selectivity two orders of magnitude higher than that of **14a**.

To determine whether cysteine alkylation prior to click chemistry with probe **14b** would reduce non-specific protein labeling by disulfide probes, we evaluated a reduction-alkylation protocol widely used in mass spectrometry. Disulfide bonds were reduced with β-mercaptoethanol and the reduced samples were alkylated with iodoacetamide. Using Western blot analysis, we compared the biotinylation signal from COS7 cell lysates incubated for 2 h in either methionine or azidohomoalanine (Aha, **21**) and then alkylated, or not, prior to click chemistry with probe **14b**. Addition of the alkylation step resulted in substantial reduction of the biotin signal in the methionine sample. The biotinylation signal from the Aha sample, however, was not changed by the alkylation step, suggesting that most, if not all, of the biotinylation signal in Aha samples is derived from conjugation to Aha residues (Figure 4). The orientation of the click reaction, (i.e., whether the alkyne functionality was on the probe or on the protein), did not affect the efficiency of the click chemistry (see supporting information).

Labeled proteins **19a-e** were subjected to cleavage conditions to yield **20a-e** (Scheme 6). Previously reported conditions for cleavage of each motif were used.[21-23] From a comparison of streptavidin signals before (samples **19a-e**) and after (samples **20a-e**) cleavage, the cleaving efficiency for each probe was calculated as a percentage of the initial streptavidin signal that remained after cleavage (Figure 3 and Table 1). Probe **9** was the least efficiently cleaved (see figure 3, lane 6b) with 23.5 ± 5.7% of the streptavidin signal remaining after one treatment with 50 mM $Na_2S_2O_4$ for 1 h. Successive treatments with $Na_2S_2O_4$ might have resulted in more complete cleavage,[30] but we were interested in comparing the probes after a single exposure to the cleaving reagent. The streptavidin signal remaining after cleavage was less than 3% for all of the other probes (**6**, **12**, **14a**, **16**). Remarkably, cleavage of the DADPS probe **12** was efficient even upon treatment with 10% formic acid. In contrast, the other acid-cleavable probe (**16**) required treatment with 95% trifluoroacetic acid.

The acid sensitivity of probe **12** was evaluated by treatment of labeled GFP with aqueous solutions of formic acid of increasing concentration. Use of 10% HCO_2H for 0.5 h at room temperature resulted in >98% cleavage (Figure 5). Treatment with 5% HCO_2H cleaved the probe with 95% efficiency, see Table 1). No significant cleavage of probe **12** was observed upon treatment with aqueous KF solutions of concentrations up to 5 M (data not shown).

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DADPS probe **12** was examined further with the objective of developing optimized procedures for enrichment of Hpg-tagged proteins. A GFP variant containing seven Hpg residues was used in this experiment to enable direct observation of labeled amino acid residues by mass spectrometry.[31] The protein was treated with probe **12** under the conditions recommended by Finn and coworkers for copper-catalyzed azide–alkyne bioconjugation.[32] After successful click reaction, two additional proteins were added to serve as positive and negative controls for binding to the streptavidin resin. Bovine serum albumin (BSA) was used as negative control to assess the removal of non-specifically bound proteins from the resin. Biotinylated horseradish peroxidase (HRP) was used as a positive control for resin binding, and also served to test if treatment with 5% formic acid would elute endogenous biotinylated proteins commonly found in cellular proteomes. After click chemistry, excess **12** was removed with a PD10 salt exchange column, and proteins were resuspended in phosphate buffered saline (PBS) at pH 7.4 with 0.1% SDS and treated with streptavidin beads. After binding, the beads were washed sequentially with 1% SDS in PBS, 6 M urea in 250 mM ammonium bicarbonate and 1 M NaCl in PBS to remove non-specifically bound proteins. The last wash contained 0.1% SDS in water to remove remaining salts from the resin before acid elution. Probe **12** was cleaved by incubating the resin in 5% formic acid in water for 2 h. The resin was washed twice with water containing 0.1% SDS to remove acid and four times with 1% SDS in PBS. These elution fractions were combined. Finally the resin was boiled for 10 min in 2% SDS in PBS.

Figure 6A shows the results of the affinity purification of 7-Hpg-GFP from a solution containing BSA and biotinylated HRP. As expected, 7-Hpg-GFP treated with probe **12** was bound to the resin throughout all washes, and then efficiently recovered after treatment with acid. Subsequent boiling of the resin with 2% SDS in PBS released no additional 7-Hpg-GFP, indicating that the majority of the protein was eluted following acid cleavage of the probe. In contrast, most of the biotinylated HRP was recovered only by boiling the resin in 2% SDS. These results show that Hpg-tagged proteins can be separated cleanly from both non-specifically bound (i.e., non-biotinylated) and endogenously biotinylated proteins.

Probe **12** was used to enrich Hpg-tagged GFP from a bacterial cell lysate. 7-Hpg-GFP was added to a lysate of *E. coli* strain DH10B cells; the lysate was then subjected to conjugation with probe **12**. The wash conditions described above were used to remove nonspecifically bound proteins; the remaining bound proteins were then eluted with 5% formic acid. Figure 6B shows the result. The GFP lane shows the position of GFP before probe conjugation. The elution lane shows clean enrichment of GFP from the complex lysate. After boiling the resin with 2% SDS we did not observe elution of more GFP, indicating efficient cleavage of **12** and elution of proteins upon treatment with 5% formic acid. The absence of significant bands corresponding to lysate proteins in both the elution fraction indicates the specificity of the enrichment strategy.

Tandem mass spectrometry was used to verify directly the conjugation and cleavage of probe **12**. Peptides resulting from tryptic digestion of eluted proteins were desalted by HPLC prior to analysis by mass spectrometry. Peptides derived from the control protein (7-Met-GFP) showed mass shifts of +16 daltons, consistent with oxidation of methionine. Peptides derived from 7-Hpg-GFP showed shifts of +121 daltons, corresponding to replacement of methionine by Hpg and subsequent conjugation and cleavage of **12** at Hpg sites (see supplementary information). The use of formic acid to cleave probe **12** works well for mass spectrometry by reducing sample complexity and the need for gel extraction. The small mass modification resulting from attachment and cleavage of the probe can serve as an aid in identification of Hpg-tagged proteins.

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Conclusion

Five cleavable biotin probes were synthesized and their protein labeling selectivity and cleaving efficiencies were compared. Probe **12**, designed around the acid sensitive dialkoxydiphenylsilane (DADPS) motif, was found to be the most selective. Efficient cleavage under mild conditions (10% formic acid, 0.5 h) and the small (143 Da) molecular fragment left on the labeled protein following cleavage make this probe especially attractive for use in proteomic studies. Probes **6**, **9**, **14** and **16** labeled less selectively than **12** in the experiments reported here, but may prove useful in analyses of other protein populations. The selectivity of the disulfide probes **14** was improved by a simple reduction-alkylation protocol that is compatible with proteomic analysis by mass spectrometry. The reactive probes described here are azides because we are interested in bioconjugation through the azide-alkyne cycloaddition, but the results should be of general interest with respect to the design of probes with other reactive groups used in biomolecular labeling studies.

Experimental Section

Synthesis

General—Unless stated otherwise, reactions were performed in flame-dried glassware under an argon or nitrogen atmosphere using dry solvents. Commercially obtained reagents were purchased from Sigma-Aldrich and used as received, unless otherwise noted. NHS-LC-biotin was obtained from Toronto Research Chemicals, homopropargylglycine (**1**) was purchased from Chiralix Inc., compounds **10** and **13** were obtained from Pierce Protein Research Products and compound **15** was obtained from Applied Biosystems. Thin-layer chromatography was performed using Baker-Flex silica gel IB-F precoated plates and visualized by UV fluorescence quenching, potassium permanganate, ceric ammonium molybdate staining or, when the biotin moiety was present, with *p*-dimethylaminocinnamaldehyde[33] staining solution. EMD Silica gel 60 (particle size 0.040-0.063 mm) was used for flash chromatography. ^{1}H and ^{13}C NMR spectra were recorded on Varian instruments and chemical shifts are reported relative to Me4Si. Data for ^{1}H NMR spectra are reported as follows: chemical shift (ppm), integration, multiplicity and coupling constant (Hz). Multiplicity and qualifier abbreviations are as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, comp = complex, br = broad, app = apparent. Low- and high-resolution mass spectra were obtained from the Mass Spectrometry Facility in the Division of Chemistry and Chemical Engineering at Caltech.

1-(5-Methyl-2-nitrophenyl)ethanone (4)—Compound **3**[24] (3.51 g, 19.6 mmol), *N*-bromosuccinimide (3.66 g, 20.6 mmol), and benzoyl peroxide (475 mg, 1.96 mmol) were refluxed in $CC1_4$ (50 mL) overnight. Benzoyl peroxide (238 mg, 0.98 mmol) was added. After 8 h, the precipitate was filtered off, the filtrate was evaporated under reduced pressure and the residue was dissolved in EtOAc. The organic phase was washed with saturated $NaHCO_3$ and with brine, and dried over Na_2SO_4. After the solvent was evaporated under reduced pressure, purification by silica gel chromatography using a 10 to 20% gradient of EtOAc in hexane gave the benzylic bromide (3.61 g, 75%); ^{1}H NMR (300 MHz, $CDCl_3$), δ (ppm): 8.13-7.91 (1H, m), 7.62 (1H, ddd, J = 20.21, 8.50, 2.00 Hz), 7.49-7.37 (1H, m), 4.55-4.41 (2H, m), 2.65-2.37 (3H, m); ^{13}C NMR (75 MHz, $CDCl_3$), δ (ppm): 199.3, 144.8, 138.4, 131.0-124.8 (m, 4C), 77.2, 30.3; *m/z* calcd for $C_9H_8BrNO_3$ $[M+H]^+$: 257.9766, MS found: 257.0, HRMS found: 257.9773. The benzylic bromide (2.00 g, 7.75 mmol) was dissolved in acetone:H_2O (5:1 by volume, 50 mL). Sodium azide (756 mg, 11.6 mmol) was added and the mixture was heated to 75 °C overnight in a flask equipped with a reflux condenser. After acetone evaporation under reduced pressure, the aqueous phase was extracted with EtOAc, washed with brine and dried with Na_2SO_4, and the solvent was evaporated under reduced pressure. Purification by silica gel chromatography using a 10 to

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20% gradient of EtOAc in hexane gave compound **4** (1.65 g, quant); ^{1}H NMR (300 MHz, $CDCl_3$), δ (ppm): 8.10 (1H, d, *J* = 8.44 Hz), 7.54 (1H, dd, *J* = 8.44, 1.92 Hz), 7.35 (1H, d, *J* = 1.89 Hz), 4.52 (2H, s), 2.61-2.50 (3H, m); ^{13}C NMR (75 MHz, $CDCl_3$), δ (ppm): 199.5, 142.9, 138.4, 129.6-124.6 (m, 4C), 53.3, 30.1; *m/z* calcd for $C_9H_8N_4O_3$ $[M+H]^+$: 221.0675, MS found: 211.3, HRMS found: 221.0679.

1-(5-(Aminomethyl)-2-nitrophenyl)ethanol (5)—Compound **4** (1.50 g, 6.81 mmol) was dissolved in MeOH:dioxane (3:2 by volume, 30 mL) and $NaBH_4$ (378 mg, 10 mmol) was added slowly. After 30 min, water (50 mL) and 2M HCl (1 mL) were added and the suspension was extracted twice with EtOAc, washed with brine, dried over Na_2SO_4 and evaporated under reduced pressure and light protection. The resulting brown oil was purified by silica gel chromatography using a 10 to 20% gradient of EtOAc in hexane to give the desired alcohol as a pale yellow oil (1.38 g, 91%); ^{1}H NMR (300 MHz, $CDCl_3$), δ (ppm): 7.89 (1H, dd, *J* = 12.06, 8.10 Hz), 7.79 (1H, dd, *J* = 8.48, 5.24 Hz), 7.36 (1H, dd, *J* = 8.37, 2.00 Hz), 5.42 (1H, dd, *J* = 12.64, 6.33 Hz), 4.47 (2H, s), 1.63-1.43 (3H, m); ^{13}C NMR (75 MHz, $CDCl_3$), δ (ppm): 141.8, 127.4-124.9 (m, 5C), 65.5, 53.9, 24.3; *m/z* calcd for $C_9H_{10}N_4O_3$ $[M+H]^+$: 223.0831, MS found: 222.6, HRMS found: 223.0831. To a solution of the alcohol (1.00 g, 4.54 mmol) in THF (30 mL), PPh_3 (1.31 g, 5.00 mmol) and H_2O (0.5 mL) were added and the mixture was heated at 60 °C for 4 h. Evaporation of the solvent under reduced pressure gave a residue that was dissolved in CH_2Cl_2 and purified by silica gel chromatography with 5-15% MeOH in CH_2Cl_2 to give compound **5** (612 mg, 70%). ^{1}H NMR (300 MHz, $CDCl_3$), δ (ppm): 7.92-7.72 (2H, m), 7.30 (1H, dd, *J* = 8.35, 1.88 Hz), 5.39 (1H, q, *J* = 6.32, 6.32, 6.32 Hz), 4.01-3.83 (2H, m), 1.67-1.37 (3H, m); ^{13}C NMR (75 MHz, $CDCl_3$), δ (ppm): 147.9, 142.2, 126.1-124.4 (m, 4C), 64.8, 45.2, 24.4; *m/z* calcd for $C_9H_{12}N_2O_3$ $[M+H]^+$: 197.0926, MS found: 196.8, HRMS found: 197.0931.

1-(2-Nitro-5-((6-(5-((3a*S*,4*S*,6a*R*)-2-oxo-hexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido)hexanamido)methyl)phenyl)ethyl 3-azidopropylcarbamate (6) —To a solution of NHS-LC-biotin (150 mg, 0.330 mmol) in DMF (5 mL), compound **5** (78 mg, 0.40 mmol) was added followed by Et_3N (69 μL, 0.50 mmol). The reaction mixture was stirred overnight at room temperature, the solvent was evaporated under reduced pressure and the residue was taken up in CH_2Cl_2 and purified by silica gel chromatography with a 5 to 15% gradient of MeOH in CH_2Cl_2 to give the desired amide (144 mg, 81%). *m/z* calcd for $C_{25}H_{38}N_5O_6S$ $[M+H]^+$: 536.2543, MS found: 536.2. To a solution of the amide (117 mg, 0.218 mmol) in DMF (5 mL), *N*,*N′*-disuccinimidyl carbonate (84 mg, 0.33 mmol) and Et_3N (91 μL, 0.66 mmol) were added. The next morning, 3-azidopropylamine (110 mg, 1.09 mmol) was added and the mixture was stirred at room temperature for 24 h. The solvent was evaporated under reduced pressure; the residue was taken up in CH_2Cl_2 and purified by silica gel chromatography using a 5 to 10% gradient of MeOH in CH_2Cl_2. An impurity that gave a white spot with DACA staining was eluted with 8% MeOH, and the desired compound **6** (101 mg, 70%) was eluted with 10% MeOH. ^{1}H NMR (300 MHz, MeOH), δ (ppm): 7.99-7.86 (1H, m), 7.61 (1H, s), 7.38 (1H, dd, *J* = 8.44, 1.74 Hz), 6.16 (1H, dd, *J* = 10.82, 4.39 Hz), 4.48 (2H, dd, *J* = 9.05, 5.91 Hz), 4.29 (1H, dd, *J* = 7.88, 4.44 Hz), 3.55-3.03 (6H, m), 3.03-2.81 (1H, m), 2.69 (1H, d, *J* = 12.74 Hz), 2.48 (1H, dd, *J* = 10.93, 4.01 Hz), 2.42-2.33 (1H, m), 2.28 (2H, t, *J* = 7.45, 7.45 Hz), 2.18 (2H, dd, *J* = 12.35, 5.04 Hz), 1.88-1.26 (17H, m); ^{13}C NMR (75 MHz, MeOH), δ (ppm): 174.9, 173.0, 170.3, 164.9, 156.6, 146.7, 145.8, 139.1, 127.1, 125.9, 124.6, 68.3, 62.2, 60.4, 55.9, 42.3, 39.9, 39.0, 37.9, 36.6, 35.7, 30.8, 29.0, 28.9, 28.6, 28.5, 28.3, 28.0, 26.4, 25.8, 25.5, 21.3; *m/z* calcd for $C_{29}H_{43}N_9O_7S$ $[M+H]^+$: 662.3084, MS found: 684.3 (M+Na), HRMS found: 662.3072.

***N*-(3-Azidopropyl)-4-((2-hydroxy-5-(2-(6-(5-((3a*S*,4*S*,6a*R*)-2-oxo-hexahydro-1*H*-thieno[3,4-*d*]imidazol-4-**

yl)pentanamido)hexanamido)ethyl)phenyl)diazenyl)benzamide (9)—To a solution of compound **8**[23] (1.50 g, 2.96 mmol) and 3-azidopropylamine (355 mg, 3.55 mmol) in CH_2Cl_2 (50 mL) was added EDC (850 mg, 4.43 mmol). The next morning, the organic phase was washed twice with 1M HCl (50 mL) and once with brine, and dried over Na_2SO_4. The solvent was evaporated under reduced pressure to give a brown oil that was dissolved in DMF (25 mL). Piperidine (2.5 mL) was added and the next morning the solvent was evaporated under reduced pressure. The residue was taken up in CH_2Cl_2 and purified by silica gel chromatography using a 10 to 50% gradient of MeOH in CH_2Cl_2 to give the desired amine (220 mg, 20% for two steps). *m/z* calcd for $C_{18}H_{22}N_7O_2$ $[M+H]^+$: 368.1835, MS found: 368.0, HRMS found: 368.1823. To a solution of NHS-LC-biotin (75 mg, 0.17 mmol) in DMF (5 mL), the above-described amine (73 mg, 0.20 mmol) was added followed by Et_3N (35 μL, 0.25 mmol). The next morning, the solvent was evaporated under reduced pressure and the residue was taken up in CH_2Cl_2 and purified by silica gel chromatography with a 5 to 10% gradient of MeOH in CH_2Cl_2 to give compound **9** (92 mg, 80%). ^{1}H NMR (300 MHz, MeOH), δ (ppm): 8.82-8.55 (m, 1H), 8.02 (m, 4H), 7.93-7.79 (m, 1H), 7.79-7.65 (m, 1H), 7.56 (s, 1H), 7.42-7.16 (m, 1H), 7.16-6.89 (m, 1H), 6.39 (d, J = 19.06 Hz, 2H), 4.39-4.18 (m, 1H), 4.18-3.99 (m, 1H), 3.71-3.15 (m, 4H), 2.95 (m, 4H), 2.62 (m, 6H), 2.01 (m, 4H), 1.79 (m, 2H), 1.34 (m, 8H); ^{13}C NMR (75 MHz, MeOH), δ (ppm): 173.5, 172.6, 166.3, 163.4, 154.2 153.6, 138.9, 136.9, 135.0, 131.6-129.1 (m, 5C), 123.1, 118.9, 61.7, 59.9, 56.1, 49.2, 37.5, 36.1, 35.9, 34.8, 29.1-28.7 (m, 4C), 26.8, 26.0-25.8 (m, 3C); *m/z* calcd for $C_{34}H_{46}N_{10}O_5S$ $[M+H]^+$: 707.3452, MS found: 731.1 (M+Na), HRMS found: 707.3462.

***N*-(2-Hydroxy-2-methylpropyl)-1-(5-((3a*S*,4*S*,6a*R*)-2-oxo-hexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido)-3,6,9,12-tetraoxapentadecan-15-amide (11)—**To a solution of compound **10** (150 mg, 0.255 mmol) in CH_2Cl_2 (5 mL), 1-amino-2-methyl-propan-2-ol (27 mg, 0.31 mmol) was added followed by Et_3N (42 μL, 0.31 mmol). After 4 h, the solvent was evaporated under reduced pressure and the residue was taken up in CH_2Cl_2 and purified by silica gel chromatography with a 15 to 20% gradient of MeOH in CH_2Cl_2 to give compound **11** (121 mg, 85%). ^{1}H NMR (300 MHz, $CDCl_3$), δ (ppm): 7.20 (s, 1H), 7.00 (s, 1H), 6.56 (s, 1H), 5.89 (s, 1H), 5.31 (s, 1H), 4.52 (s, 1H), 4.34 (d, J = 4.58 Hz, 1H), 3.85-3.51 (m, 20H), 3.51-3.36 (m, 3H), 3.26 (d, J = 5.96 Hz, 2H), 3.15 (d, J = 4.36 Hz, 1H), 2.89 (d, J = 4.78 Hz, 1H), 2.52 (t, J = 5.84, 5.84 Hz, 2H), 2.24 (t, J = 7.41, 7.41 Hz, 2H), 1.67 (dd, J = 14.92, 7.45 Hz, 4H), 1.42-1.09 (m, 8H); *m/z* calcd for $C_{25}H_{46}N_4O_8S$ $[M+H]^+$: 563.3115, MS found: 563.2, HRMS found: 563.3089.

***N*-(4-((6-Azidohexyloxy)diphenylsilyloxy)butyl)-1-(5-((3a*S*,4*S*,6a*R*)-2-oxo-hexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido)-3,6,9,12-tetraoxapentadecan-15-amide (12)—**To a solution of dichlorodiphenylsilane (0.19 mL, 0.88 mmol) and Et_3N (0.34 mL, 2.4 mmol) in anhydrous CH_2Cl_2 (20 mL) was added compound **11** (0.10 g, 0.18 mmol). After 10 h, 6-azidohexanol (0.30 g, 2.2 mmol) was added. The solvent was evaporated 16 h later, and the residue was taken up in CH_2Cl_2 and passed through a silica gel column with 10% MeOH in CH_2Cl_2. The solvents were evaporated under reduced pressure and the residue dissolved in CH_2Cl_2 was washed with a saturated solution of $NaHCO_3$. The $NaHCO_3$ solution was extracted with CH_2Cl_2 three times. The CH_2Cl_2 fractions were combined and dried over Na_2SO_4. After removal of CH_2Cl_2 under reduced pressure, a second purification by silica gel chromatography with a 2 to 10% gradient of MeOH in CH_2Cl_2 gave compound **12** (86 mg, 55%). ^{1}H NMR (300 MHz, $CDCl_3$), δ (ppm): 7.62 (dd, J = 7.74, 1.54 Hz, 4H), 7.54-7.20 (m, 6H), 6.83-6.66 (m, 1H), 6.56-6.39 (m, 1H), 5.71-5.47 (m, 1H), 4.54-4.35 (m, 1H), 4.35-4.17 (m, 1H), 3.87-3.45 (m, 20H), 3.41 (dd, J = 5.57, 2.80 Hz, 3H), 3.31 (d, J = 5.98 Hz, 2H), 3.22-3.06 (m, 2H), 2.96-2.78 (m, 1H), 2.72 (s, 1H), 2.40 (t, J = 6.07, 6.07 Hz, 2H), 2.19 (d, J = 7.34 Hz, 2H), 1.82-1.49 (m, 6H), 1.37 (m, 6H), 1.31-1.10 (m, 8H); ^{13}C NMR (126 MHz, $CDCl_3$), δ (ppm):

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173.3, 171.2, 164.0, 134.7 (m, 6C), 130.2 (m, 2C), 127.8 (m, 4C), 75.7, 70.3 (m, 8C), 67.3, 63.0, 61.8, 60.2, 55.6, 51.4, 50.4, 40.5, 39.1, 37.1, 35.9, 32.2, 28.8, 28.2, 27.6, 26.4, 25.6 (2C), 25.3; *m/z* calcd for $C_{43}H_{67}N_7O_9SSi$ $[M+H]^+$: 886.4569, MS found: 908.2 (M+Na), HRMS found: 886.4601.

N-(2-((3-(3-Azidopropylamino)-3-oxopropyl)disulfanyl)ethyl)-1-(5-((3a*S*,4*S*, 6a*R*)-2-oxo-hexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido)-3,6,9,12-tetraoxapentadecan-15-amide (14a)—To a solution of compound **13** (50 mg, 0.067 mmol) in CH_2Cl_2 (5 mL), 3-azidopropylamine (8.7 mg, 0.086 mmol) was added followed by Et_3N (12 μL, 0.086 mmol). After 2 h, the solvent was evaporated under reduced pressure and the residue was taken up in CH_2Cl_2 and purified by silica gel chromatography with a 5 to 12% gradient of MeOH in CH_2Cl_2 to give compound **14a** (39 mg, 80%). ^1H NMR (300 MHz, $CDCl_3$), δ (ppm): 7.40 (s, 1H), 7.19 (s, 1H), 7.10-7.01 (m, 1H), 6.55 (s, 1H), 5.99-5.78 (m, 1H), 4.60-4.44 (m, 1H), 4.34 (d, *J* = 4.42 Hz, 1H), 3.91-3.47 (m, 20H), 3.47-3.23 (m, 5H), 3.15 (d, *J* = 4.46 Hz, 1H), 3.00 (t, *J* = 7.00, 7.00 Hz, 2H), 2.95-2.72 (m, 3H), 2.63 (dd, *J* = 15.62, 8.69 Hz, 2H), 2.51 (t, *J* = 5.81, 5.81 Hz, 2H), 2.24 (t, *J* = 7.27, 7.27 Hz, 2H), 2.03-1.56 (m, 6H), 1.45 (d, *J* = 6.94 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$), δ (ppm): 173.6, 172.0, 171.4, 164.1, 70.2 (m 8C), 67.2, 61.8, 60.2, 55.7, 49.2, 40.5, 39.2, 38.5, 38.1, 36.8 (2C), 36.4, 35.8, 35.0, 28.7, 28.1, 25.6; *m/z* calcd for $C_{29}H_{52}N_8O_8S_3$ $[M+H]^+$: 737.3149, MS found: 759.2 (M+Na), HRMS found: 737.3148.

N-(2-((3-(propargylamino)-3-oxopropyl)disulfanyl)ethyl)-1-(5-((3a*S*,4*S*,6a*R*)-2-oxo-hexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido)-3,6,9,12-tetraoxapentadecan-15-amide (14b)—The procedure described above for the preparation of compound **14a** was followed except that 3-azidopropylamine was replaced by propargylamine, and afforded **14b** in 81% yield. ^1H NMR (500 MHz, $CDCl_3$), δ (ppm): 6.97 (s, 1H), 6.35 (s, 1H), 5.52 (s, 1H), 4.57-4.44 (m, 1H), 4.33 (dd, *J* = 7.29, 4.87 Hz, 1H), 4.05 (dd, *J* = 5.22, 2.49 Hz, 2H), 3.75 (t, *J* = 5.81, 5.81 Hz, 2H), 3.69-3.60 (m, 16H), 3.56 (m, 4H), 3.44 (dd, *J* = 7.15, 3.78 Hz, 2H), 3.20-3.07 (m, 2H), 3.03-2.97 (m, 2H), 2.91 (dd, *J* = 12.80, 4.88 Hz, 1H), 2.85 (t, *J* = 6.51, 6.51 Hz, 2H), 2.75 (d, *J* = 12.76 Hz, 1H), 2.65 (t, *J* = 7.17, 7.17 Hz, 2H), 2.51 (t, *J* = 5.81, 5.81 Hz, 2H), 2.37-2.16 (m, 4H), 1.82-1.60 (m, 4H), 1.43 (td, *J* = 21.27, 7.37, 7.37 Hz, 2H), 1.25 (s, 1H); ^{13}C NMR (126 MHz, $CDCl_3$), δ (ppm): 173.5, 172.0, 171.0, 163.9, 80.0, 71.3, 70.2 (m 8C), 67.3, 61.8, 60.2, 55.6, 45.9, 40.6, 39.2, 38.4, 36.9, 36.0, 34.4, 29.1, 28.1, 25.6, 8.6; *m/z* calcd for $C_{29}H_{49}N_5O_8S_3$ $[M+H]^+$: 692.2822, MS found: 714.2 (M+Na), HRMS found: 692.2822.

Probe 16—To a solution of **15** (0.5 mg, 0.45 μmol) in acetonitrile (1 mL), 3-azidopropanethiol (85 μg, 0.7 μmol) was added followed by Et_3N (0.10 μL, 0.7 μmol). The next morning, the solvent was evaporated and H_2O (1 mL) was added. Lyophilization of the sample gave **16** (approx 0.5 mg, quant.). *m/z* calcd for $C_{48}H_{85}N_{11}O_9S_2$ $[M+H]^+$: 1024.6051, MS found: 1024.1, HRMS found: 1024.6043.

General protocol for click chemistry—Protein **17** or **18** (100 μL of a 0.50 mg/mL 50% glycerol stock solution) was added to 800 μL of PBS, pH 7.8, followed by SDS (100 μL of a 10% w/v stock solution). The biotin probe (10 μL of a 5 mM stock solution in DMSO) and the Tris-[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine ligand (10 μL of a 20 mM stock solution in DMSO) were added followed by a freshly prepared and vigorously vortexed CuBr solution (20 μL of a 3 mg CuBr/mL in H_2O stock solution, made from CuBr 99.999% purchased from Sigma-Aldrich). The mixture was stirred vigorously at 15 °C for 4 h, added dropwise to a centrifugation tube containing acetone (5 mL) and kept at -20°C for 2 h. Centrifugation at 2000*g* for 10 min at 4 °C yielded a pellet that contained protein and copper; the pellet was resuspended by sonication in 100 μL PBS, pH 7.8, containing 3%

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SDS. Centrifugation at 2000*g* for 10 min at 4 °C sedimented the copper in the form of a blue pellet. The supernatant containing the labeled protein was stored at -20 °C until needed.

General protocol for probe cleavage—The reagent required for cleavage of each probe was added to the corresponding labeled protein sample at room temperature. After the desired reaction time (see Table 1), aliquots were mixed with SDS gel loading buffer, loaded on an SDS gel and subjected to western blot analysis as described below.

Western blot analysis—Tris-glycine SDS gels (12% acrylamide) were run (without pre-boiling the samples) using a Fisher Scientific FB3000 instrument at 165V/250mA for 45 min. Proteins were transferred to a GE Healthcare Life Science Hybond ECL membrane using a Hoefer Scientific Instruments TE50X at 80 V for 1 h at 4°C. After the membrane was blocked with 5% milk powder in PBS, pH 7.4, containing 0.5% TWEEN 20, the residual milk solution was removed by three washes with 10 mL of PBS, pH 7.4, containing 0.5% TWEEN 20. Anti penta-his alexa fluor 488 conjugate (Qiagen, 2 μL, 1:5000) and streptavidin alexa fluor 633 conjugate (Invitrogen, 4 μL, 1:2500) were added to the membrane in 10 mL of PBS, pH 7.4, containing 0.5% TWEEN 20. The membrane was kept in the dark under gentle shaking for 45 min. The residual antibodies were washed five times with 10 mL of PBS, pH 7.4, containing 0.5% TWEEN 20; fluorescence was measured on a GE Healthcare Typhoon Trio™ Variable Mode Imager.

Cloning—The previously reported expression vector pQE-80/GFPrmAM[30] was subjected to site-directed mutagenesis to generate the M13L mutation of GFPrmAM, yielding pJS2. pJS2 encodes a GFP variant that contains only one methionine site, at the N-terminus. Constructs were confirmed by sequencing.

Protein expression and purification—pJS2 was transformed into the methionine auxotrophic *E. coli* strain M15MA/pREP4 and isolated on LB-agar plates containing 35 μg/mL kanamycin and 200 μg/mL ampicillin. A single colony was used to inoculate an overnight 5 mL culture in LB containing kanamycin and ampicillin. The following morning, 1 mL of the culture was diluted in 49 mL of M9 minimal medium (M9 salts, 0.2 % glucose, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 25 mg/mL thiamine) and supplemented with 40 mg/L of each of the twenty natural amino acids in addition to kanamycin and ampicillin. Cells were grown until OD_{600} reached 1.0 (4-5 h), pelleted at 2000 *g* for 10 min at 4°C and washed twice in cold, sterile 0.9% NaCl. Cells were resuspended in M9 (-Met) medium (M9 medium supplemented with 40 mg/L of each of the natural amino acids except methionine). Either Hpg or methionine was added to a final concentration of 1 mM. IPTG was added to a final concentration of 1 mM to induce synthesis of GFP. Cells were induced for 4 h at 37°C and pelleted at 2000 *g* for 10 min at 4°C; the supernatant was removed and cells were frozen at -80°C overnight. The His-tagged GFP was purified on a Qiagen NiNTA resin under native conditions according to the manufacturer's recommendations. The purified protein was subjected to buffer exchange into PBS, pH 7.8, on GE Healthcare PD10 columns according to the manufacturer's protocol. Glycerol was added to the protein sample at a final concentration of 50%, and the protein was stored in 50% glycerol at -20°C until used.

Protein digestion and mass spectrometry—GFP samples (20 μg) were dissolved in 8 M urea in 100 mM Tris-HCl, pH 8.5, and incubated in 3 mM Tris(2-carboxyethyl)phosphine hydrochloride for 20 min at room temperature. The samples were then treated with 11.5 mM iodoacetamide for 15 min, followed by Endoprotease Lys-C (Promega) digestion according to the manufacturer's recommendations. The sample was diluted to 2 M urea with 100 mM Tris-HCl, pH 8.5, and adjusted to 1 mM $CaCl_2$. Tryptic digestion (Promega) was carried out according to the manufacturer's recommendations, and was followed by addition of 5%

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formic acid to stop trypsin activity. The resulting peptides were desalted by HPLC (Waters Corp.) on a C8 peptide microtrap (Michrom Bioresources, Inc.). The peptides were analyzed on a Thermo Finnigan Orbitrap FT mass spectrometer in positive ion mode with collision-induced dissociation (CID) for ms/ms analysis.

Iodoacetamide alkylation—COS7 cells were grown to 80% confluence in DMEM++ media. Cells were washed and incubated for 30 min in Hepes-buffered saline, pH 7.4 (HBS), followed by 2 h incubation in either 4 mM methionine in HBS or 4 mM Aha in HBS. Cells were pelleted and lysate was prepared as described previously.[17] Cell lysate was reduced in 2% β-mercaptoethanol in PBS pH 7.8 for 1 h in the dark at 25°C and then proteins were precipitated in acetone. Upon resuspension in 250 μL PBS, pH 7.8, proteins were alkylated by treatment with 11 mM iodoacetamide for 1 h in the dark at 25°C, and then again precipitated in acetone. After a second resuspension in 500 μL PBS pH 7.8, the click chemistry reaction was assembled as described previously.[17]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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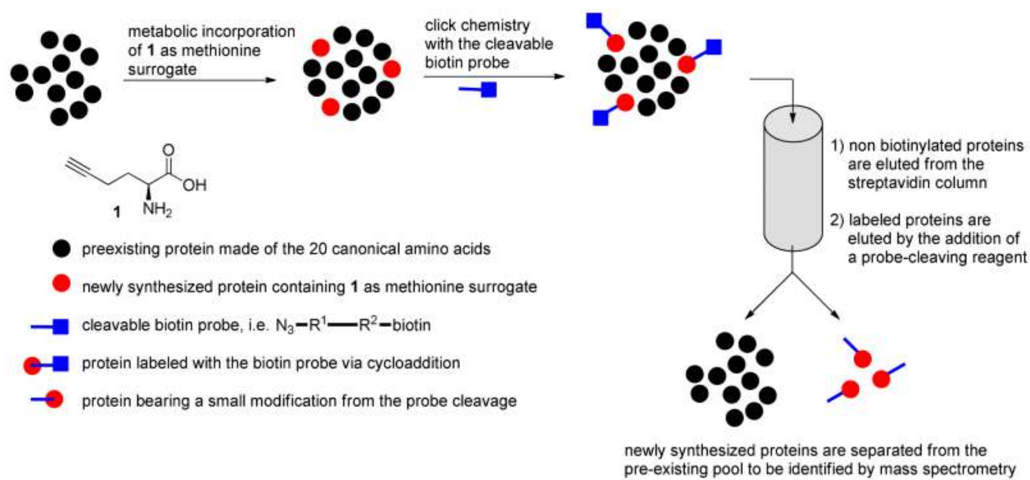

Figure 1.
Schematic representation of the BONCAT method.

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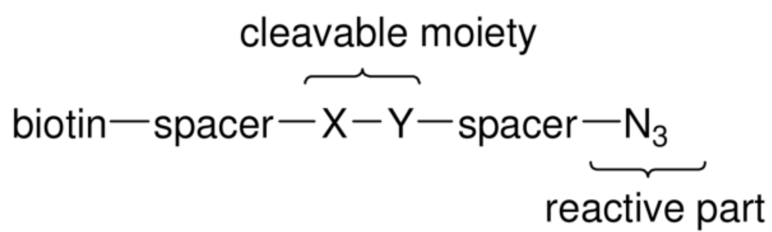

Figure 2.
Elements of a generic cleavable biotin probe.

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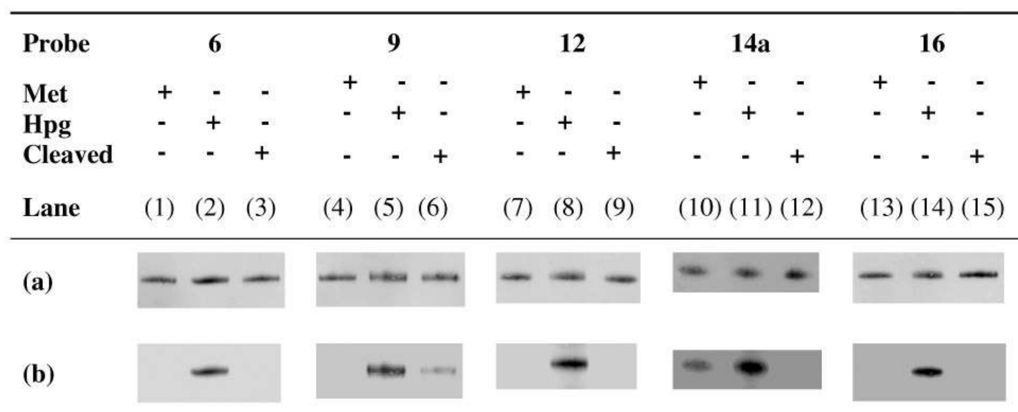

Figure 3.

Anti-His (a) and streptavidin (b) signals to determine the labeling and cleaving efficiencies of each probe. Lane 1: **17** + **6** treated with click conditions; lane 2: **19a**; lane 3: **20a**; lane 4: **17** + **9** treated with click conditions; lane 5: **19b**; lane 6: **20b**; lane 7: **17** + **12** treated with click conditions; lane 8: **19c**; lane 9: **20c**; lane 10: **17** + **14a** treated with click conditions; lane 11: **19d**; lane 12: **20d**; lane 13: **17** + **16** treated with click conditions; lane 14: **19e**; lane 15: **20e**.

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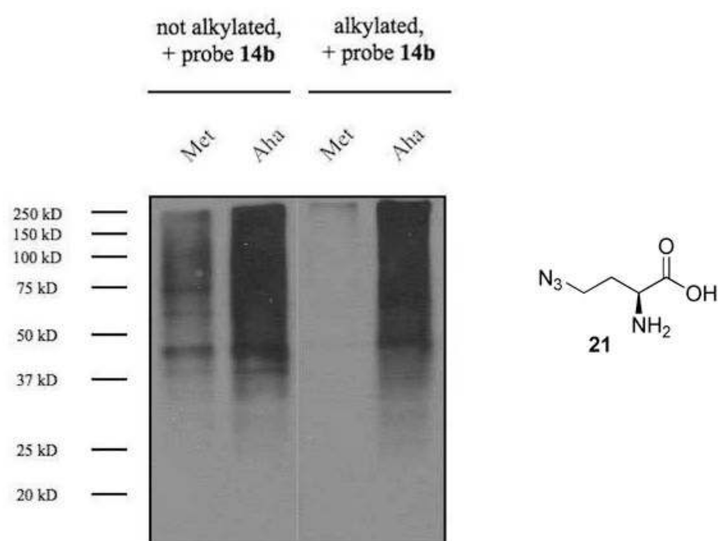

Figure 4.

Alkylation prior to click chemistry significantly reduces non-specific protein labeling by probe **14b**. COS7 cells incubated in either methionine or Aha were lysed and either not alkylated (lanes 1 and 2) or alkylated (lanes 3 and 4) prior to reaction with **14b**. Western blots were visualized using an antibody against biotin.

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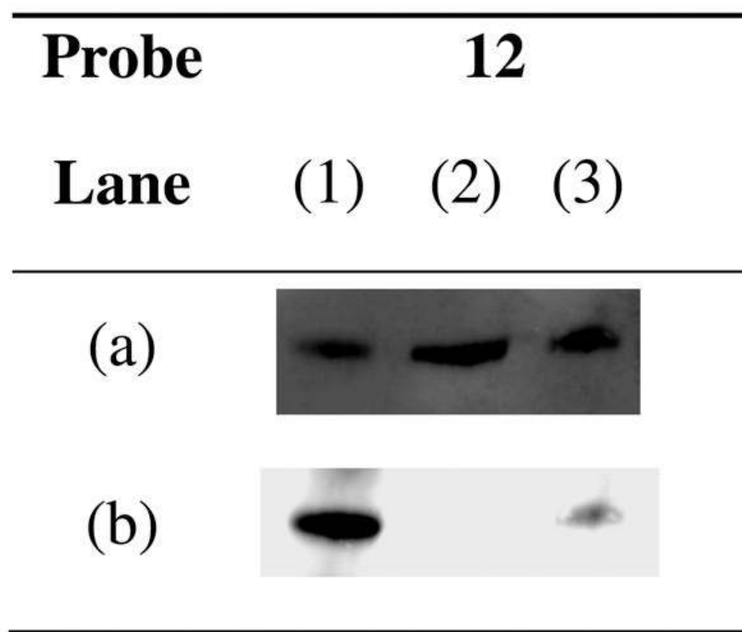

Figure 5.

Anti-His (a) and streptavidin (b) signals for the cleavage of probe **12** with 10% and 5% formic acid. Lane 1: **19c** not treated with any acid after 5 h; lane 2: **19c** treated with 10% HCO_2H for 0.5 h; lane 3: **19c** treated with 5% HCO_2H for 0.5 h.

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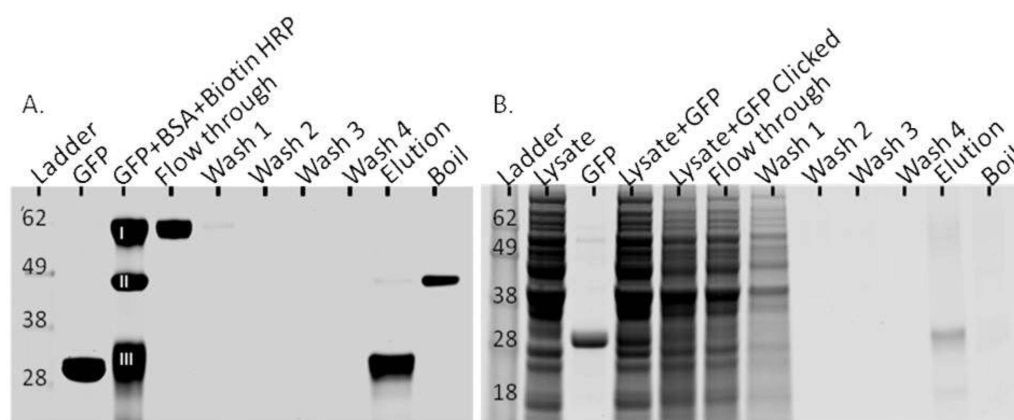

Figure 6.

Polyacrylamide gel analysis of affinity purification of 7-Hpg-GFP. A: After click reaction with probe **12**, affinity purification from a solution containing BSA (band I), biotinylated HRP (band II), and 7-Hpg-GFP (band III), was performed on a streptavidin agarose resin. Wash 1 was performed with 1% SDS in PBS; wash 2 with 6 M urea in 250 mM ammonium bicarbonate; wash 3 with 1 M NaCl in PBS and wash 4 with 0.1% SDS in water to remove PBS. Elution with 5% formic acid was followed by boiling of resin in 2% SDS in PBS to remove remaining proteins. B: Affinity purification of 7-Hpg-GFP from a DH10B lysate. Wash and elution conditions were identical to those in Figure 6A. Gels were stained with Coomassie Brilliant Blue.

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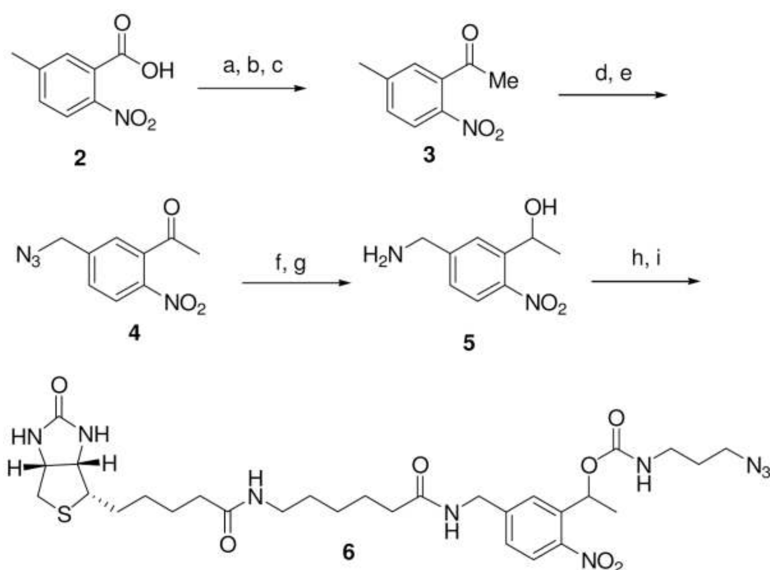

Scheme 1.
Synthesis of a photocleavable biotin probe.
Reagents and conditions: a) $SOCl_2$; b) Mg, diethyl malonate; c) HCl, 83% for 3 steps; d) NBS, $(BzO)_2$, 75%; e) NaN_3, quant.; f) $NaBH_4$, 91%; g) PPh_3, H_2O, 70%; h) NHS-LC-biotin, 80%; i) DSC then 3-azidopropylamine, 70%.

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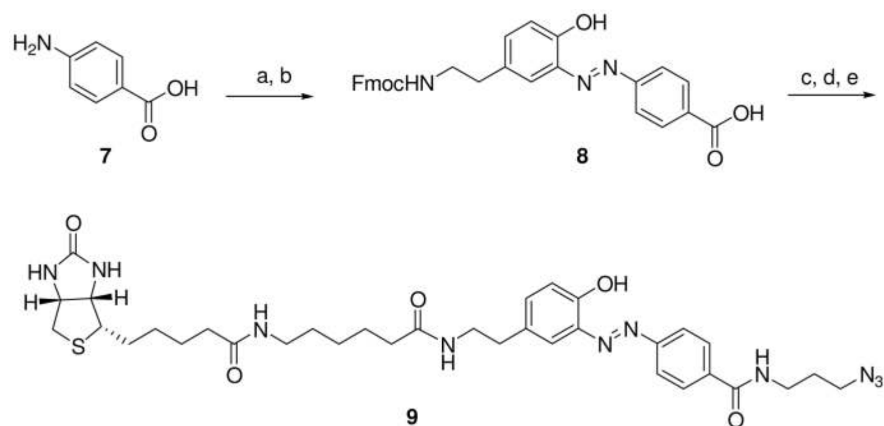

Scheme 2.

Synthesis of a $Na_2S_2O_4$ cleavable biotin probe.

Reagents and conditions: a) $NaNO_2$, HCl; b) tyramine, $NaHCO_3$ then Fmoc-Cl, 55% for 2 steps; c) EDC, 3-azidopropylamine; d) piperidine, 20% for 2 steps; e) NHS-LC-biotin, 80%.

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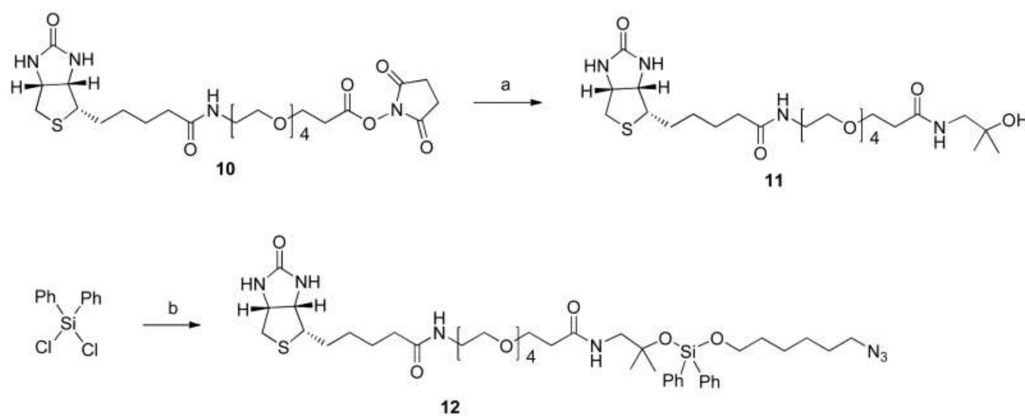

Scheme 3.

Synthesis of the DADPS biotin probe.

Reagents and conditions: a) 1-amino-2-methyl-propan-2-ol, 85%; b) Et_3N, DCDPS then 6-azidohexanol, 55%.

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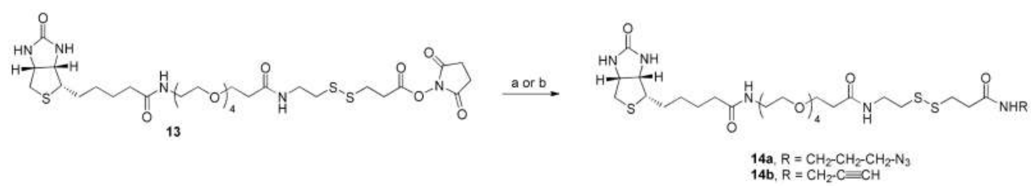

Scheme 4.
Synthesis of disulfide biotin probes.
Reagents and conditions: a) 3-azidopropylamine, 80%; b) propargylamine, 81%.

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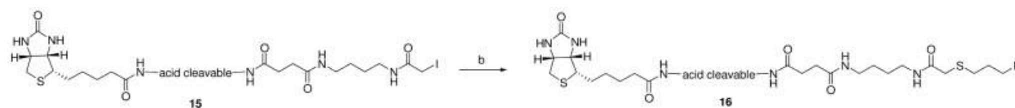

Scheme 5.
Synthesis of the acid cleavable biotin probe.
Reagents and conditions: a) 3-azidopropanethiol, Et_3N, quant.

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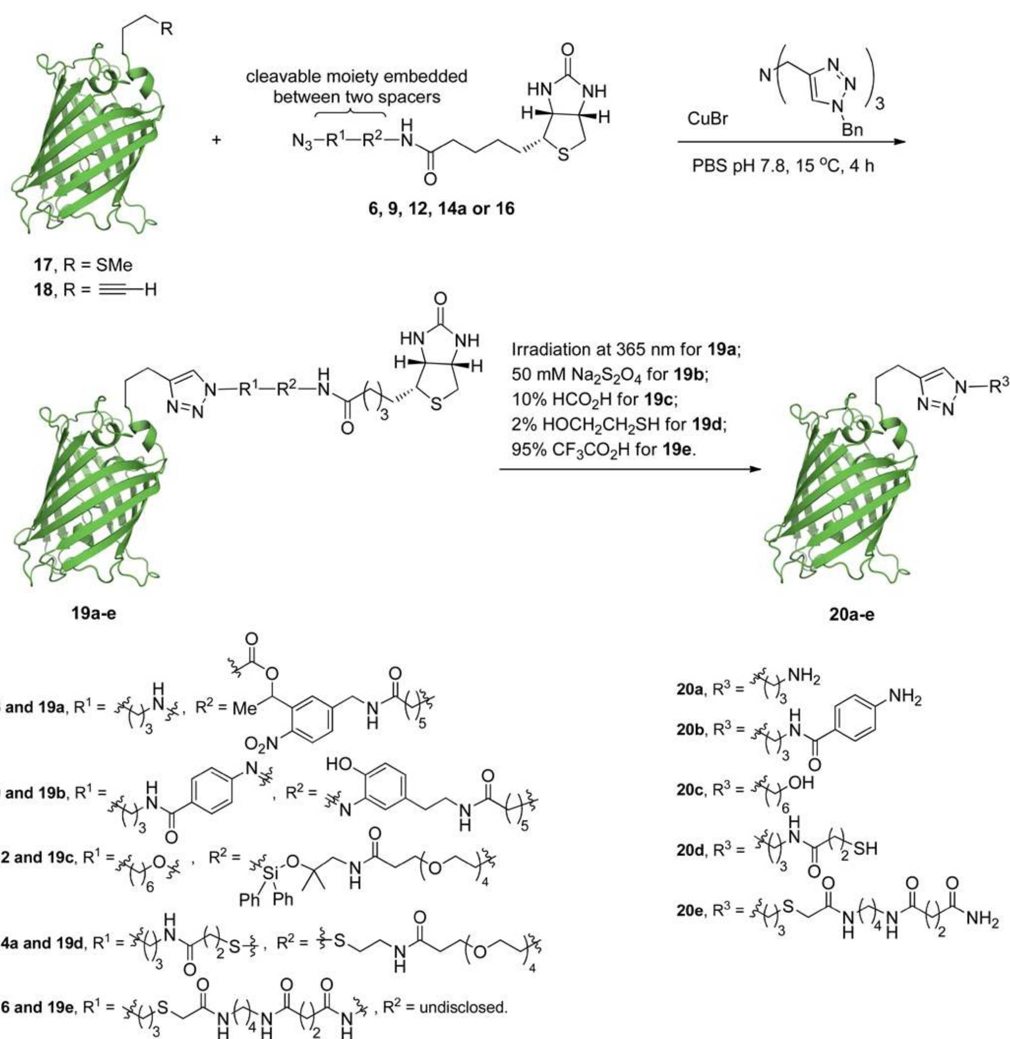

Scheme 6.

Protein labeling and probe cleavage.

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Table 1

Labeling and cleaving efficiencies calculated for each probe.[a]

Probe	6	9	12	14a	16
Relative selectivity[b]	14.5 ± 2.2	14.7 ± 3.4	117.5 ± 18.3	1.0 ± 0.2[c]	10.8 ± 2.5
Biotin remaining after cleavage (%)	1.1 ± 0.4	23.5 ± 5.7	1.6 ± 0.4 (5.4 ± 1.3)	2.4 ± 0.3	2.5 ± 0.9
Cleaving conditions	hν 365 nm 30 min	$Na_2S_2O_4$ 50 mM, 1 h	10% HCO_2H, 0.5 h (5% HCO_2H, 0.5 h)	$HO(CH_2)_2SH$ 2%, 1 h	CF_3CO_2H 95%, 2 h
MW left behind (Da)[d]	100	219	143	188	344

[a]All streptavidin and anti-His fluorescence signals were measured on a GE Healthcare Typhoon Trio™ Variable Mode Imager by integrating the peak corresponding to the green fluorescent protein molecular weight. All streptavidin signals were normalized to the corresponding anti-His signals. All numbers are based on triplicate analysis.

[b]Relative selectivity = (**19a-e** streptavidin signal ÷ **19a-e** anti-His signal)/(**17** streptavidin signal ÷ **17** anti-His signal), where the signals associated with **17** are those observed after treatment with the cleavable probe.

[c]**14a** relative selectivity was arbitrarily designated as unity.

[d]MW = molecular weight.