Chapter 5

IMAGING NEWLY SYNTHESIZED PROTEINS IN LIVING CELLS

5.1   Abstract

Proteins tagged with azide-containing amino acids or sugars can be selectively modified by alkyne-containing fluorophores in complex cellular environments. Many of the azide-reactive coupling partners reported thus far are membrane impermeant and require a toxic copper catalyst, making the visualization of intracellular proteins in live cells difficult. We have synthesized a series of fluorescent coumarin-cyclooctyne conjugates and tested their ability to label intracellular proteins tagged with the methionine analogue azidohomoalanine in Rat-1 fibroblasts. Confocal fluorescence micrographs showed that all four fluorophores labeled intracellular proteins; the non- and monofluorinated cyclooctyne dye-labeling was localized to the cytoplasm, while the more reactive difluorinated cyclooctyne dye showed both cytoplasmic and nuclear staining. The increase in fluorescence over background ranged from 8- to 20-fold, as measured by flow cytometry using concentrations of 10 or 50 μM, depending on the dye. Staining with MitoTracker Red and propidium iodide indicated that cells remained viable during labeling and imaging. These coumarin-cyclooctyne conjugates provide a powerful new tool for visualizing protein dynamics inside living cells.

5.2 Introduction

Fluorescent labeling of proteins allows the visualization of dynamic cellular processes. Labeling is most often accomplished through the generation of chimeric proteins by fusing the protein of interest to a fluorescent protein, such as GFP.[1, 2] This strategy has been employed successfully for imaging live cells. However, the addition of a large (30 kDa) tag can cause unpredictable perturbations in the target protein's localization and activity. An alternative approach involves the introduction of a short peptide tag to the protein of interest that recognizes a small molecule fluorescent probe.[3-5] These methods are powerful tools for biological studies of pre-selected proteins, but suffer from the drawback of requiring genetic manipulation as their means of introduction. This prevents their ready use in studies of global cellular processes, such as protein synthesis or post-translational modification.

In the post-genomic era, a more complete understanding of biological systems will be attained through strategies that tag proteins based on their spatial, temporal, or chemical identity. Cellular proteins can be specifically tagged with small, reactive metabolite analogues by co-opting the host's metabolic machinery. This strategy has been employed to tag proteins with ketone,[6-9] azide,[10-13] or alkyne[14-16] functionalities to enable selective, minimally invasive protein tagging in complex biological mixtures. Glycosylated,[17-19] phosphorylated,[20] farnesylated,[21] and fatty-acylated[22, 23] proteins have been isolated after incorporation of reactive analogues. These post-translational modifications are regulated dynamically, and metabolic tagging has enabled proteome-level analyses that were not accessible by genetic manipulation.

Similarly, reactive amino acid analogues can be incorporated directly into proteins by using the cell's translational machinery, enabling the selective modification of newly synthesized proteins.[24-28]   The azide- and alkyne-containing methionine (Met) analogues azidohomoalanine (Aha) and homopropargylglycine (Hpg) have been used to both visualize and identify a defined subset of the proteome.[29, 30] The use of these reactive analogues is reminiscent of conventional pulse-labeling with radioactive amino acids. In the present application, the endogenous cellular machinery places the reactive Met surrogates, rather than radioactive derivatives, at sites normally occupied by Met within proteins. Proteins containing Aha and Hpg can then be reacted with azide- or alkyne-containing ligation partners using the highly efficient and selective copper-catalyzed azide-alkyne ligation.[31, 32]   While this reaction has been used successfully for labeling purified proteins, complex cellular extracts, and fixed cells, the toxicity of copper makes this method incompatible with living cells[33, 34].   In contrast, the Staudinger ligation[11, 35] and the strain-promoted azide-alkyne ligation[33, 36-38] are compatible with living cells. In this chapter, we describe the use of a strain-promoted ligation to label newly synthesized proteins with a set of coumarin-cyclooctynes.

5.3   Results and Discussion

We hypothesized that the more biologically compatible strain-promoted azide-alkyne ligation reaction could be used to dye label Aha-containing proteins inside healthy, living cells. The biocompatible ligation of cyclooctynes to azidosugars has been explored previously[33].   Further investigation demonstrated that the reactivity of the cyclooctyne could be improved by the addition of one or two fluorine atoms adjacent to

the alkyne[36, 38]. The improved reactivity of the fluorinated cyclooctynes enables rapid and sensitive labeling of cell surface azides for dynamic cell imaging and has been used to monitor glycan trafficking[36]. We anticipated that the conjugation of coumarin, a small, membrane-permeant fluorophore, to a cyclooctyne moiety would result in a molecule that could both be taken up by live cells and enable ligation to azides displayed on newly synthesized proteins.   Commercially available dimethylaminocoumarin (DMAC) was conjugated to various differentially-activated cyclooctyne acids via a short linker segment using standard coupling chemistry to give the coumarin-cyclooctyne conjugates **1-4** (**Scheme 5.1**). The linker segment was included to improve the solubility of the dye and to increase the distance between the dye and its protein target, which may can improve fluorescence signal[39].

These reactive coumarin-cyclooctynes were assessed for their ability to label newly synthesized proteins inside a mammalian Rat-1 fibroblast cell line using confocal fluorescence microscopy (**Figures 5.1** and **5.2**). Cells were treated for 4 h with either 1 mM Aha, 1 mM Met, or 1 mM Aha pre-treated with the protein synthesis inhibitor
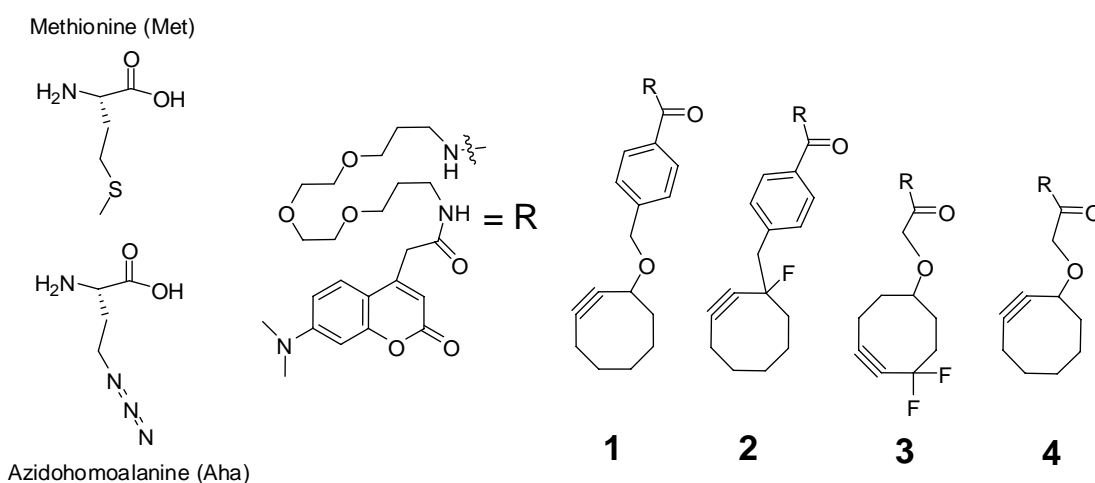
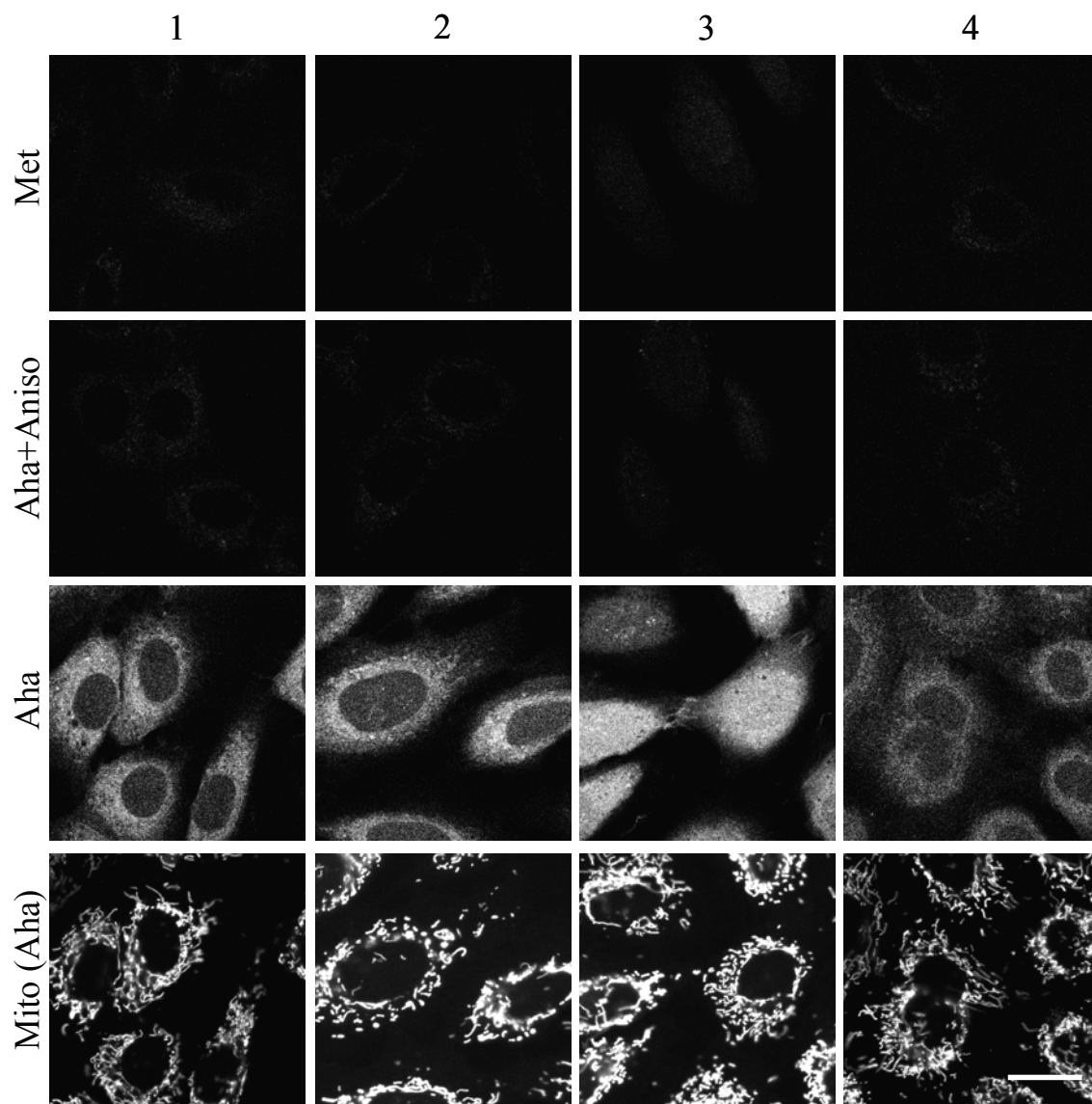**Scheme 5.1.** Structure of Met, Aha, and the coumarin-cyclooctynes.

Figure 5.1. Fluorescence labeling of proteins in Rat-1 Fibroblasts. Confocal fluorescence imaging of fibroblasts grown for 4 h in media containing 1 mM Met (top row), 1 mM Aha pre-treated with the protein synthesis inhibitor anisomycin (aniso; second row), or 1 mM Aha (bottom two rows). After the pulse, cells were dye-labeled for 10 min with 50 μM **1** (column 1), 50 μM **2** (column 2), 10 μM **3** (column 3), or 50 μM **4** (column 4). Cells were counterstained with MitoTracker Red before imaging. The images for each dye (**1**, **2**, **3**, or **4**) were acquired using identical conditions to capture either coumarin or MitoTracker Red fluorescence. Images of the mitochondria for cells pulsed with Aha are included to help distinguish individual cells as well as the cytoplasmic space [(Mito(Aha)]. The scale bar represents 20 μm.
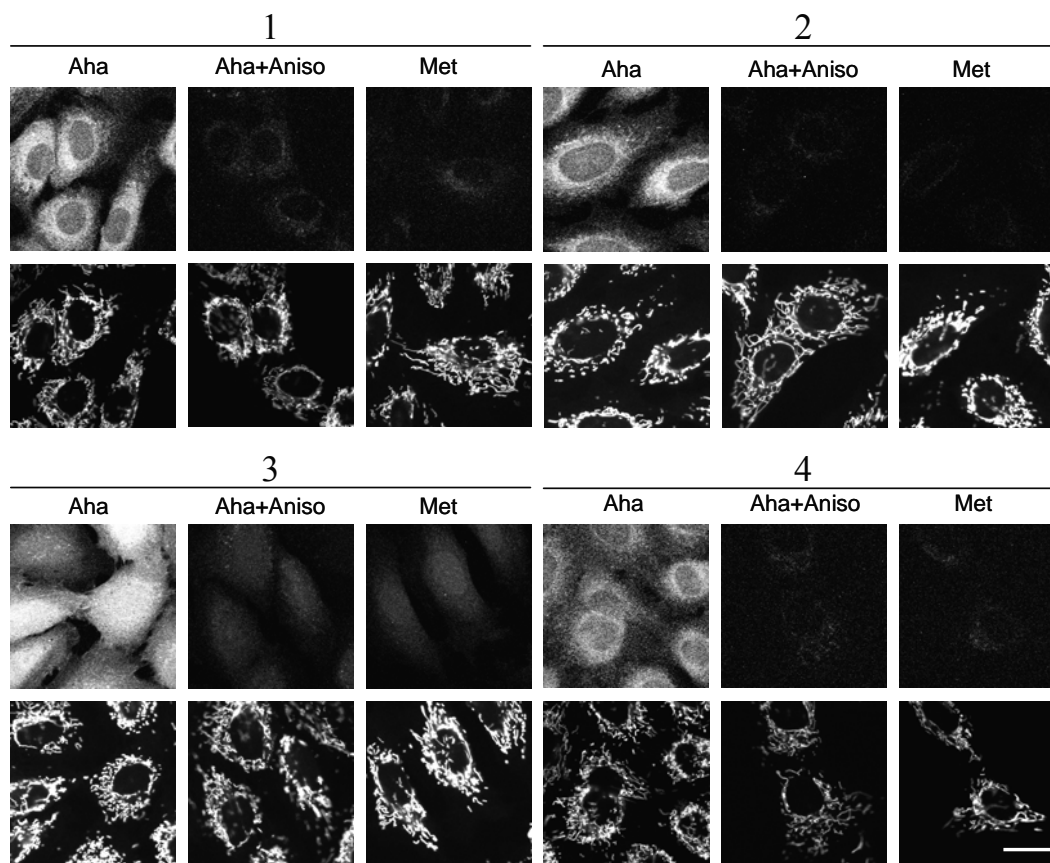
Figure 5.2. Projections of coumarin fluorescence from labeled proteins in Rat-1 Fibroblasts. The coumarin projections correspond to the single slice coumarin images shown in **Figure 5.1**. Single slice images of the mitochondria are included to distinguish individual cells and the cytoplasmic space and to ensure correct mitochondrial morphology. The scale bar represents 20 μm.

anisomycin ([Aha + aniso]). The [Aha + aniso] control was included as a means to assess the contribution of free Aha to overall fluorescence signal in the cells. After the 4 h pulse, cells were washed and incubated with one of the dye conjugates for 10 min at 37 °C. Following incubation, the cells were washed again and treated with MitoTracker Red (Invitrogen), a membrane-permeant dye that localizes to functional mitochondria. The

MitoTracker Red counterstain aids in delineating individual cells, distinguishing the cytoplasmic space from the nucleus, and assessing mitochondrial morphology as a measure of cell health. After counterstaining, cells were washed a final time and examined using confocal microscopy. Images were captured using identical conditions for cells exposed to Aha, [Aha + aniso], or Met. Substantial fluorescent labeling of cells was observed after 10 min of treatment with **1-4**. The fluorescence of cells treated with Aha alone was much brighter than that of cells treated with either Met or [Aha + aniso], indicating that the observed labeling is specific to Aha and that most of the coumarin-labeled Aha is protein associated.

Unexpectedly, the fluorescence images revealed a distinction in the cellular localization of the dye conjugates. Fluorescence in Aha-treated cells was bright in the cytoplasm and dim in the nucleus for **1** and **2**, while the interior of cells labeled with **3** appeared more uniformly fluorescent (**Figures 5.1 and 5.2**). The molecular structure of **3** differs from the other dye conjugates in two distinct ways: **3** is more highly fluorinated than either **1** or **2,** and the linker segment in **3** is shorter and lacks the benzene ring contained in **1** and **2**. We sought to understand what feature of **3** was responsible for the difference in intracellular localization by synthesizing a dye conjugate with a reduced linker length (**4**, **Scheme 5.1**). Dye conjugate **4** exhibited dim nuclear staining similar to that observed for **1** and **2**, and we concluded that the nuclear staining for **3** can be attributed to the additional fluorination, either through the improved reactivity that this imparts to the conjugate or through a less well-defined change in the hydrophobic character of the dye.[36]

We utilized flow cytometry to determine a set of optimal conditions for labeling

newly synthesized proteins as a function of the coumarin-cyclooctyne concentration, the

reactive amino acid pulse length, and fluorophore-labeling time. Cells were pulse-labeled

for 4 h with Aha or Met before treatment with each of the conjugates **1-4** for 10 min over

a range of concentrations between 0.5 to 50 μM of each dye (**Figure 5.3**). The mean

coumarin fluorescence increased with increasing dye concentration for all four dyes. The

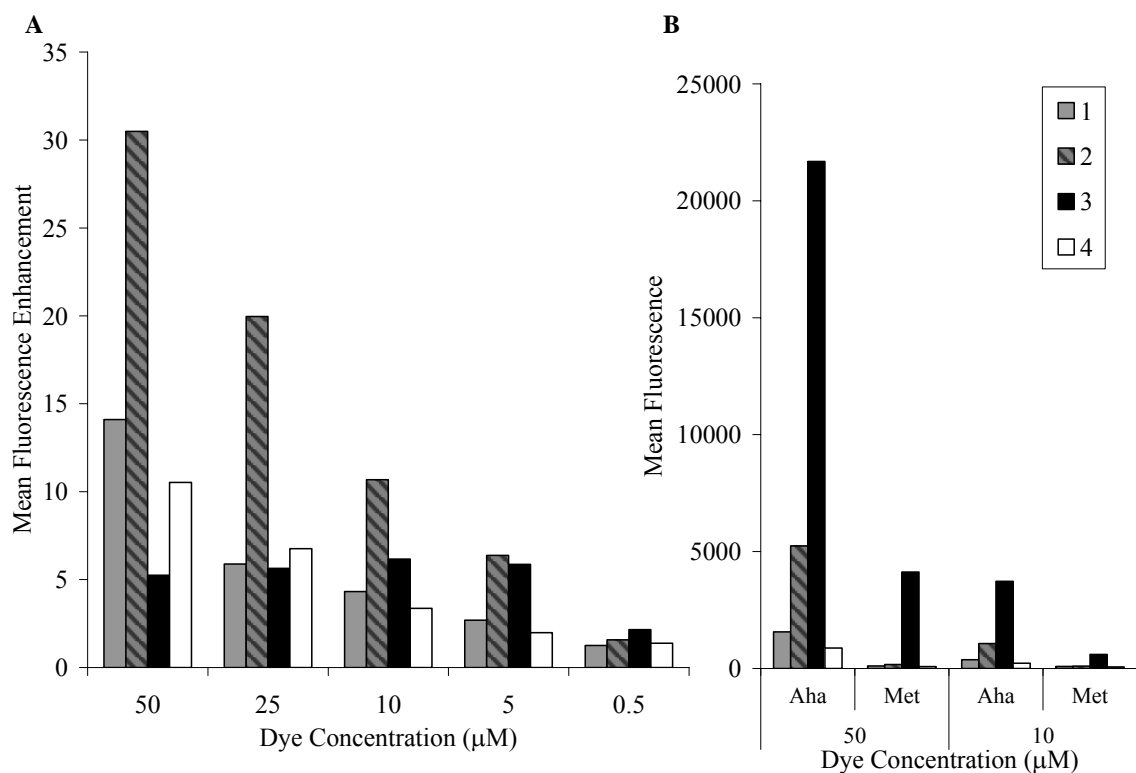fluorescence enhancement, defined as the mean fluorescence for Aha-treated cells

Figure 5.3. Flow cytometric analysis of coumarin fluorescence as a function of dye-concentration for cells

pulse-labeled 4 h with Aha or Met. **A.** Mean fluorescence enhancement for cells dye-labeled for 10 min (**1**

grey bars, **2** hashed bars, **3** black bars, and **4** white bars). **B.** Mean fluorescence values for cells dye-

labeled 10 min with either 10 μM or 50 μM of each dye. For each sample, 20,000 events were collected.

divided by the mean fluorescence for Met-treated cells, increased with increasing concentration for **1**, **2**, and **4**. In contrast, the optimal enhancement for cells treated with **3** was observed not at the highest dye concentration, but at 10 μM **3**.

We hypothesized that the high cellular labeling for cells pulse-labeled with Met and dye-labeled with **3** might be attributed to significant side reactions with cellular nucleophiles. We examined the specificity of **3** using liquid chromatography to monitor the reaction between 1 μM **3** and 1 mM amino acid (Aha, Met, or cysteine) in PBS (pH 7.4) at 37 °C (**Figure 5.4**). Unreacted **3** and the products were observed by detecting coumarin absorbance at 350 nm. By monitoring the decrease in peak area of **3** over time, we were able to confirm that the *in vitro* reaction between Aha and **3** is rapid, with a half-life of 16 ± 3 min. No reaction was observed with Met, even after 24 h. Next, we looked at the reaction between the biological nucleophile cysteine (Cys) and **3**. The *in vitro* reaction between **3** and Cys was slower than the reaction of **3** with Aha, but still proceeded at a significant rate with a half-life of 27 ± 5 min. Although enhancements in labeling can clearly be obtained with **3**, side reactions resulting from the enhanced reactivity of this dye should not be discounted when labeling cells with this fluorophore.

The optimal pulse length for the reactive amino acids prior to exposure to coumarin-cyclooctynes was determined by examining four time intervals (15 min, 30 min, 1 h, and 4 h; **Figure 5.5a**). The longer pulses (1 h and 4 h) with Aha gave the largest enhancements, but labeling could be achieved with shorter pulse lengths. Aha pulses of 30 min gave a 4-fold enhancement in mean fluorescence for **2** (50 μM) or a 2-fold enhancement with **3** (10 μM). More modest enhancements (1.3- to 1.5-fold) were observed after a 15 min Aha pulse and treatment with 10 μM **3**. Further adjustments to

the system, considering factors such as local application of the reactive amino acid, increasing the concentration of amino acid, or utilizing different cell lines might reveal better conditions for using shorter pulse lengths effectively.

We used a pulse length of 4 h and a labeling concentration of 10 μM dye to examine the effect of dye labeling time on the system (**Figure 5.5b**). Cells were examined after exposure to each dye for periods of 6, 10, 30, or 60 min. After 10 min of dye labeling, cells expressing proteins containing Aha show enhancements in fluorescence. Modest labeling was also observed at 6 min, the shortest labeling time examined. Labeling for as long as 60 min offered further increases in mean fluorescence
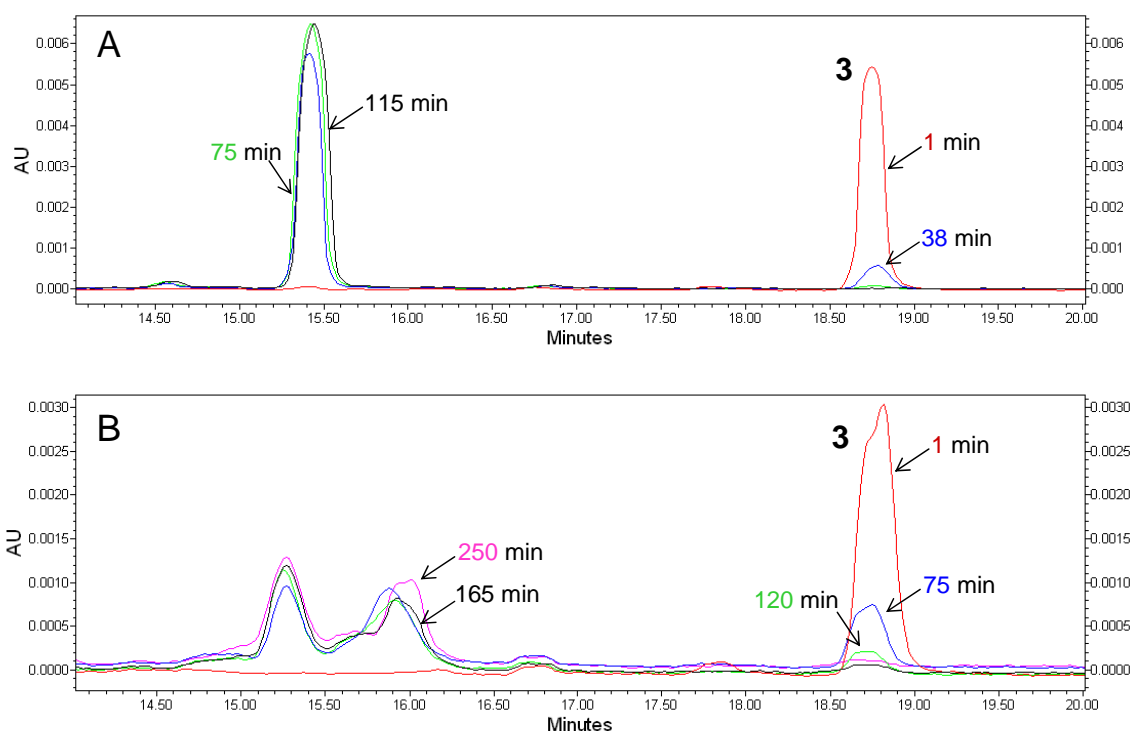

Figure 5.4. Chromatograms of the *in vitro* reaction of **3** with Aha or Cys. Reaction mixtures contained 1 μM **3** with 1 mM Aha (**A**) or 1 mM Cys (**B**) in PBS (pH 7.4). The reactions proceeded at 37 °C until analysis by HPLC. Detection at 350 nm was used to observe changes in the starting material (**3**: 18.5-19 m) and product peaks (Aha: 15-16 m, Cys: 14-17 m) at different time points.
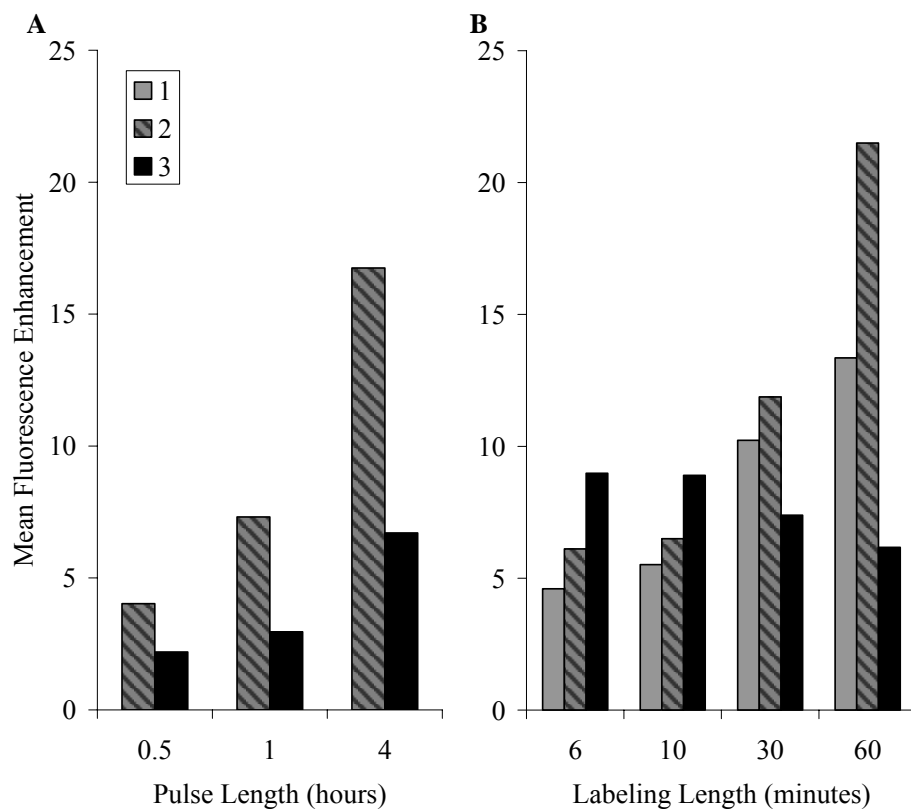
Figure 5.5. Flow cytometric analysis of pulse-labeling length and dye-labeling length. **A.** Mean fluorescence enhancement of cells pulse-labeled for 0.5, 1, or 4 h. Cells were dye-labeled with 10 μM **3** or 50 μM **2** for 10 min before analysis. **B.** Mean fluorescence enhancement of cells labeled with 10 μM **1**, **2** or **3** for different times after a 4 h pulse. For each sample, 30,000 events were collected.

for samples stained with **1** and **2**, but the non-specific fluorescence-labeling with **3** became substantial. For **1** and **2**, short labeling times can be combined with increased dye concentration (50 μM) to achieve higher fluorescence enhancements (data not shown).

The extent of fluorescent labeling under the optimized conditions for each of the four dyes was quantified using flow cytometry. After a 4 h Aha pulse, cells were dye-labeled for 10 min with 10 μM **3** or 50 μM **1**, **2**, or **4** (**Figure 5.6**). Cells treated with Aha

and labeled with 10 μM **3** displayed a mean fluorescence that was 10-fold higher than for the Met control cells. Cells treated with 50 μM **1**, **2,** or **4** gave enhancements of 15-fold, 20-fold, and 8-fold, respectively. Exposure to anisomycin prior to pulse-labeling with Aha gave fluorescence levels comparable to those observed for cells treated with Met.

In order to gain useful biological information from labeling experiments, it is necessary to minimize the extent to which a labeling method perturbs cell metabolism and affects cell viability. Three distinct methods confirmed that exposure of cells to **1-4** and subsequent imaging did not compromise cell viability. MitoTracker Red was used to stain active mitochondria and to ensure that the mitochondrial morphology was normal during imaging. Additionally, cells were counterstained with propidium iodide to confirm that cells remained viable after labeling with the coumarin-cyclooctynes (**Figure**
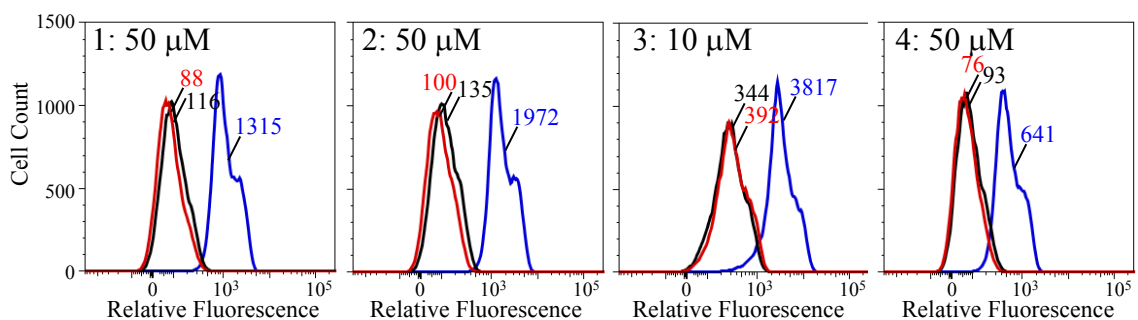

Figure 5.6. Histograms of coumarin fluorescence from flow cytometry. Cells were pulsed for 4 h in media supplemented with 1 mM Met (red), 1 mM Aha + anisomycin (aniso; black), or 1 mM Aha (blue). After the pulse, cells were dye-labeled for 10 min with 50 μM **1**, 50 μM **2**, 50 μM **4**, or 10 μM **3**. Values given indicate the mean fluorescence for each population of cells. For each sample, 30,000 events were collected after excluding dead cells from analysis using forward-scatter, side-scatter, and 7-AAD fluorescence.
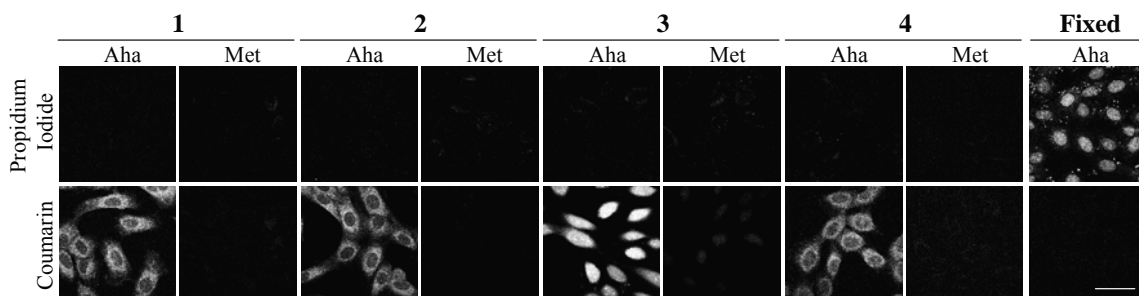
Figure 5.7. Viability of cells dye-labeled with coumarin-cyclooctynes. Cells were pulse-labeled 4 h in media supplemented with 1 mM Met or 1 mM Aha. Cells were dye-labeled 10 min with 50 μM **1**, 50 μM **2**, 10 μM **3**, or 50 μM **4**. Cells were washed, treated with 1 μg/mL propidium iodide for 10 min, washed, and then imaged. Fixed cells were treated with 3.7% paraformaldehyde for 10 min instead of staining with coumarin cyclooctyne. After fixation, cells were permeabilized and stained with propidium iodide. The scale bar represents 50 μm.

5.7). Finally, phase contrast microscopy was used to verify that cells divided and remained well-spread after imaging experiments. These three methods indicated that staining and imaging with the coumarin-cyclooctynes did not hinder the viability of the cells.

5.4   Conclusion

We have synthesized a set of coumarin-cyclooctynes which represent a new class of reactive dyes capable of labeling azide-containing proteins within living cells. After brief exposure to **1-4**, cells pulse-labeled with Aha were characterized by observable and quantifiable enhancements in fluorescence relative to cells treated with Met. The optimal conditions described in Figure 5.6 resulted in mean fluorescence enhancements of 8-fold or higher for **1-4**. Although the fluorescence micrographs indicate that **1**, **2**, and **4** label proteins predominantly in the cytoplasm, use of **3** facilitates the more uniform labeling of

proteins in both the nucleus and cytoplasm. However, the non-specific labeling by **3** should be considered when selecting a dye for cellular labeling. In an effort to access a larger region of the fluorescent spectrum, other cyclooctyne-conjugated fluorophores are being evaluated for their ability to selectively label azide-tagged biomolecules.

5.5   Materials and Methods

5.5.1   Cell maintenance

Rat-1 fibroblasts (ATCC) were maintained in a 37 °C, 5% CO_2 humidified incubator chamber. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (Invitrogen), 50 U/mL penicillin, and 50 μg/mL streptomycin (DMEM++). Near-confluent cells were passaged with 0.05% trypsin in 0.52 mM EDTA (Invitrogen).

5.5.2   Preparation of Cells for Fluorescence Microscopy

Near-confluent cells in 100 mm Petri dishes were rinsed twice with warm phosphate-buffered saline (PBS). Cells were detached with trypsin in EDTA and added to DMEM++. The cells were pelleted via centrifugation (200g, 3 min) and counted. Cells were added at a density of 1 x 10^4 cells per well to prepared slides. Cells were grown in DMEM++ overnight.

Lab-Tek chambered coverglass slides (8-well, Nalge Nunc International) were prepared by treatment with fibronectin solution (10 μg/mL). The wells were rinsed twice with PBS, blocked with a 2 mg/mL solution of heat-inactivated BSA at room temperature, and rinsed with PBS.

After growth overnight in DMEM++, each well was washed twice (200 μL) with warm PBS. Cells were incubated for 5 min in serum-free medium lacking Met [SFM: DMEM, with 1 mg/mL bovine serum albumin (BSA, fraction V, Sigma-Aldrich), with 2 mM Glutamax I (Invitrogen), without Met], followed by 30 min in fresh SFM to deplete intracellular Met stores. Anisomycin (40 μM, Sigma-Aldrich) was added to cells during this time to inhibit protein synthesis. After incubation, either 1 mM Met or 1 mM Aha was added to the medium. After 4 h, wells were rinsed twice with DMEM++ before adding the dye-labeling mixture.

Cells were exposed to coumarin-cyclooctyne in the labeling media DMEM-Imaging [DMEM lacking phenol red, with HEPES (Invitrogen), supplemented with 10% FBS and 1 mg/mL BSA]. Labeling was allowed to proceed 10 min at 37 °C in the incubator chamber. After labeling, cells were washed twice before counterstaining. Cells were counterstained for 10 min with 300 nM MitoTracker Red CMXRos (Invitrogen). After treatment, cells were washed thrice with DMEM-Imaging and then imaged in DMEM-Imaging. Cells were kept in an incubator until they could be imaged (up to 3 h).

For counterstaining with propidium iodide, cells were washed twice with DMEM++ before the addition of a 1:1000 dilution of propidium iodide (1.0 mg/mL; Invitrogen) in DMEM-Imaging for 10 min. Cells were washed thrice before imaging. Fixed (3.7% paraformaldehyde, 10 min) and permeabilized (0.1% Triton X-100 in PBS, 3 min) cells were also imaged as a control. Fixed cells stained with propidium iodide were not treated with coumarin-cyclooctyne.

5.5.3 *Preparation of Cells for Flow Cytometry*

Pulse-labeling was performed directly in the 6-well tissue culture dishes in which cells were grown. Each well was washed twice with warm PBS, followed by a 30 min incubation in SFM to deplete intracellular Met stores. Cells were then exposed to 1 mM Aha or 1 mM Met for 4 h. In addition to examining a 4 h pulse-length, we also examined 0.8, 0.25, 0.5, and 1 pulses. Then, cells were washed twice with PBS. Coumarin-cyclooctyne dye in DMEM-Imaging was added to each well for coumarin-labeling. Dye concentrations of 0.5 μM to 50 μM were examined. To examine alternative dye-labeling times, 10 μM dye was added for 6-60 min in DMEM-Imaging. After labeling, cells were washed twice with warm PBS and detached using 250 μL of 0.05% trypsin in EDTA. Cells were added to 750 μL DMEM++, and 100 μL FBS was added to the bottom of the Eppendorf tube to minimize cell losses. Cells were pelleted by centrifugation (200g, 3 min) and washed once with 1 mL DMEM-Imaging, again with a cushion of FBS added to the bottom of the tube. Lastly, cells were resuspended in 400 μL DMEM-Imaging before filtering through a 50 μm Nytex nylon mesh screen (Sefar). Cells were stored on ice until analysis.

5.5.4 *Fluorescence Microscopy*

Live cells were imaged on a confocal microscope (Zeiss LSM 510 Meta NLO) at California Institute of Technology's Biological Imaging Center. A heated chamber was placed around the microscope to image the cells at ~37 °C. MitoTracker Red or propidium iodide fluorescence was obtained by excitation at 543 nm with emission collected between 565 and 615 nm. Transmitted light images were also collected to

differentiate individual cells. Coumarin fluorescence was obtained by two-photon excitation at 800 nm (Ti:sapphire laser) with emission collected between 376 and 494 nm. The set of images for each dye was obtained with identical conditions to capture coumarin fluorescence. Individual optical slices of coumarin fluorescence were collected at 0.5 μm intervals in order to create an extended focus image (i.e., projection). Images were acquired with a Plan-Apochromat 63x/1.4 oil objective (Zeiss) and analyzed with Zeiss LSM and ImageJ software.

5.5.5 Image Processing

For MitoTracker Red, propidium iodide, and coumarin images in **Figures 5.1**, **5.2**, and **5.7**, the brightness and contrast were manually adjusted for the images corresponding to each dye using ImageJ Software. Then, the minimum and maximum pixel values were applied to each image from samples treated with that dye. Stacks of coumarin fluorescence images were made into a projection using ImageJ software. A maximum intensity projection was created by taking the maximum value at each pixel along the Z-axis of the image plane through a stack of ten 0.5 μm-thick images. Again, the brightness and contrast were manually adjusted and applied to each image in the set (Aha, Met, and Aha+anisomycin).

5.5.6 Flow Cytometry

Cells were analyzed on a FACSAria flow cytometer (BD Biosciences Immunocytometry Systems) at Caltech's Flow Cytometry Facility. Coumarin fluorescence was excited by a 407 nm laser and detected after passage through a 450/40

bandpass filter. Forward- and side-scatter properties were used to exclude doublets, dead cells, and debris from analysis. 7-aminoactinomycin D (7-AAD; Beckman Coulter) was used to exclude dead cells from analysis. 7-AAD was excited by a 488 nm laser and detected after passage through a 695/40 filter. Unlabeled cells, 7-AAD labeled cells, and coumarin labeled cells were analyzed to ensure minimal cross-over fluorescence in each channel. If necessary, compensation was applied to reduce cross-over fluorescence. Data was analyzed using FloJo7 software.

5.5.7 *Synthesis of Coumarin-Cyclooctyne Dyes*

All chemicals were purchased from Aldrich and used as received unless otherwise noted. Dry solvents were obtained from commercial suppliers and used as received. Silica chromatography was performed using 230-400 mesh silica gel 60 (EMD). TLC was run on Baker-flex silica gel IB-F plates, R_f's are reported under the same solvent conditions as columns unless otherwise noted. TLC was examined under UV light for fluorescent compounds or alternatively stained with $KMnO_4$, ceric ammonium molybdenate, or *p*-anisaldehyde. NMR spectra were recorded on Varian spectrometers (300 MHz for ^{1}H) and processed with NUTS nmr software. NMR spectra were referenced to internal standards; proton and carbon spectra were referenced to tetramethylsilane. Fluorine spectra were referenced to hexafluorobenzene. FAB mass spectrometry was performed at the California Institute of Technology Mass Spectrometry Facility.

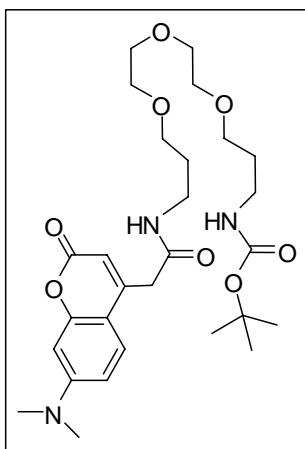Cyclooctyne acids **5** - **8** were prepared as described [33, 36, 38].
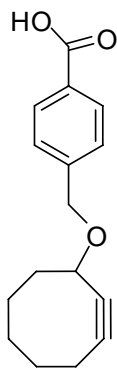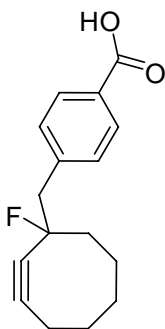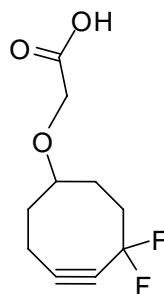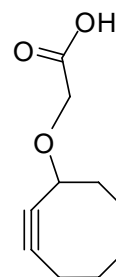
5　　　　**6**　　　　**7**　　　　**8**
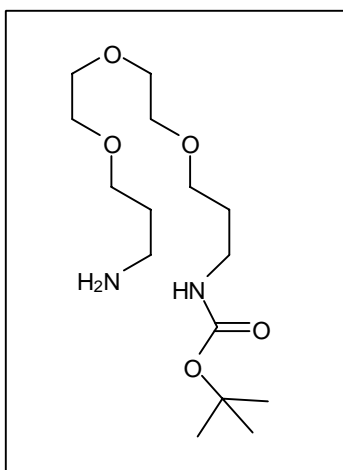
1-Boc-amino-13-amino-4,7,10-trioxotridecane, **9**. A solution of ditertbutyldicarbonate (5.0 g, 22.9 mmol) in 100 ml of CH_2Cl_2 was added via a dropping funnel to a rapidly stirred solution of 1,13-diamino-4,7,10-trioxotridecane (10.1 g, 45.8 mmol) in 200 ml CH_2Cl_2 over a period of ~1 h. The resulting clear solution was stirred 16 h then transferred to a separatory funnel and washed twice with 100 ml of 0.1 M HCl and the aqueous layer was back extracted 5 times with 50 ml of CH_2Cl_2. The combined organics were dried over $NaSO_4$ and evaporated to give 6.6 g (90%) of an opaque viscous oil, which was used without further purification. ^{1}H NMR (300 MHz, CDCl$_3$) δ 1.44 (s, 9 H),1.55 (br s, 2H), 1.68-1.84 (m, 4H), 2.81 (t, 1H, $J = 6.8$), 3.23 (br q, 2H, $J = 6.2$), 3.51-3.72 (m, 12H), 5.02-5.29 (2x br t, rotomers, 1H); ^{13}C NMR (75 MHz, CDCl$_3$) δ 28.46, 29.61, 33.26, 38.48, 39.62, 69.47, 69.56, 70.18, 70.22, 70.58, 70.60, 78.80, 156.05, 156.10; FAB MS calc for $C_{15}H_{33}N_2O_5$ (M+H) 321.2389, observed 321.2386.

Boc-Linker-Coumarin **10**. To a solution of 1-Boc-amino-13-amino-4,7, 10-trioxotridecane, **9**, (0.027g, 80 μmol) and triethylamine (0.015 g, 140 μmol) in 2 ml of CH_2Cl_2 in a foil wrapped flask was added 7-dimethylaminocoumarin-4-acetic acid, succinimidyl ester (0.025 g, 72.6 μmol, Anaspec) in 1 ml of CH_2Cl_2. The reaction was stirred 12 h and then the solvent removed and the product purified by silica chromatography (1:19 ethanol:ethyl acetate, R_f = 0.25) yielding 40 mg ~100%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.43 (s, 9 H),1.73 (app quintet, 4 H, J = 6.2),2.70 (s, 2 H),3.06 (s, 6 H),2.19 (br q, 2 H, J = 6.3),3.36 (q, 2 H, J = 6.0),3.45-3.65 (m, 12 H),5.01 (br, t, 1 H),6.07 (s, 1 H),6.50 (d, 1 H, J = 2.8),6.62 (dd, 1 H, J = 2.5, 9.0),6.75 (br t, 1 H), 7.53 (d 1 H J = 9.0); ^{13}C NMR (75 MHz, $CDCl_3$) δ, 28.44, 28.61, 29.66, 38.52, 40.12, 40.62, 69.42, 70.02, 70.08, 70.14, 70.24, 70.39, 78.89, 98.145, 108.50, 109.13, 110.24, 125.84, 150.18, 153.04, 155.98, 161.86, 168.01; FAB MS calc for $C_{28}H_{43}N_3O_8$ (M+) 549.3050, observed 549.3027.
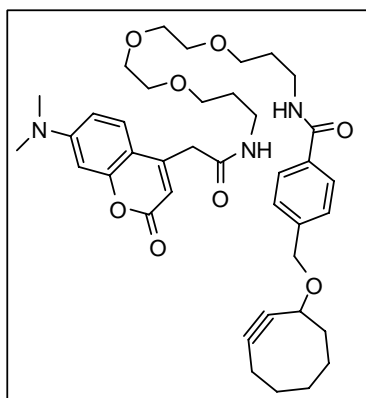
1. Cyclooctyne acid **5** (0.0245 g, 0.095 mmol) was dissolved in 2 ml of DMF in a foil wrapped flask and placed under argon. Pyridine (18 μl, 0.105 mmol) was added, and the solution was cooled in an ice bath. Pentafluorophenyltrifluoroacetate (18 μl, 0.10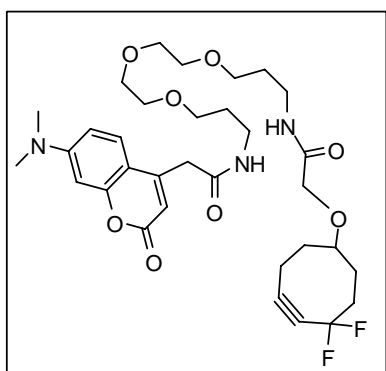5 mmol) was added, and the solution was stirred for 2 h. The solvent was diluted with ethyl acetate (20 ml), extracted twice with 1M HCl, extracted once with saturated $NaHCO_3$, dried over Na_2SO_4, and evaporated to give a clear oil that

was resuspended in 2 ml of CH$_2$Cl$_2$. This solution was added to a solution of **10** (0.043g, 0.072 mmol) that had been deprotected by treatment with 1:1 TFA:CH$_2$Cl$_2$ for 2 h, followed by solvent removal and resuspension in 2 ml CH$_2$Cl$_2$ and 22 μl (0.160 mmol) triethylamine. The combined solution was stirred under argon and monitored by TLC. After 6 h, the reaction mixture was applied directly to a silica column and eluted with 2:9:9 Ethanol:CH$_2$Cl$_2$:Ethyl Acetate (R$_f$ =0.24 in 1:19 Ethanol:CH$_2$Cl$_2$) to give 0.044 g (0.064 mmol, 88%) of a yellow-white solid. ^{1}H NMR (300 MHz, CDCl$_3$) δ 1.46 (m, 1 H), 1.59-1.76 (m, 4 H), 1.79-1.93 (m, 5 H), 1.93-2.39 (m, 4 H), 3.04 (s, 6 H), 3.27 (app q, 2 H, J = 5.9 Hz), 3.41-3.47 (m, 4 H), 3.51-3.66 (m, 12 H), 4.22 (m, 1 H), 4.16 (d, 1 H, J = 12.5), 4.69 (d, 1 H, J = 12.5), 6.06 (s, 1 H), 6.46 (d, 1 H, J = 2.4), 6.59 (dd, 1 H, J = 9.1, 2.5), 6.79 (br t, 1 H), 7.27 (br t, 1 H), 7.38 (d, 2 H, J = 8.1 hz), 7.49 (d, 1 H, J = 9.1 hz), 7.77 (d, 2 H, J = 8.1 hz);^{13}C NMR (75 MHz, CDCl$_3$) δ 20.70, 26.37, 28.53, , 28.85, 29.72, 34.30, 38.63, 38.89, 40.09, 40.49, 42.32, 53.46, 69.92, 70.06, 70.17, 70.28, 70.42, 70.46, 72.04, 92.50, 98.04, 100.66, 108.38, 109.20, 110.06, 125.76, 127.11, 127.70, 133.53, 141.81, 150.19, 153.07, 155.94, 162.12, 167.58, 168.39; FAB MS calculated for C$_{39}$H$_{51}$N$_3$O$_8$ (M+H) 690.3754, observed 690.3720.
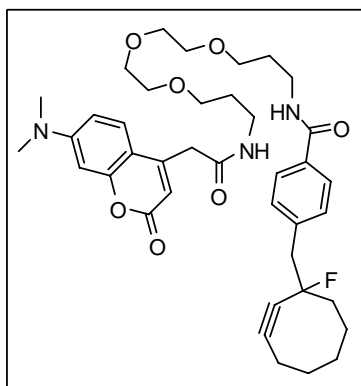
2.   Clyclooctyne acid **6** (0.005 g, 0.019 mmol) was dissolved in 0.2 ml of DMF in a foil wrapped flask and placed under argon. Pyridine (6 μl, 0.075 mmol) was added, and the solution was cooled in an ice bath. Pentafluorophenyltrifluoroacetate (4 μl, 0.023 mmol) was added, and the solution was stirred for 3 h. The solvent

was diluted with ethyl acetate (20 ml), extracted twice with 1M HCl, extracted once with saturated NaHCO$_3$, dried over Na$_2$SO$_4$, and evaporated to give a clear oil that was resuspended in 2 ml of CH$_2$Cl$_2$. This solution was added to a solution of **10** (0.020g, 0.04 mmol) that had been deprotected by treatment with 2 ml of 1:1 TFA:CH$_2$Cl$_2$ for 2 h, followed by solvent removal and resuspension in 2 ml CH$_2$Cl$_2$ and 22 µl (0.160 mmol) triethylamine. The combined solution was stirred under argon and monitored by TLC. After 8 h, the reaction mixture was applied directly to a silica column and eluted with 1:19 Ethanol:CH$_2$Cl$_2$ (R$_f$ = 0.24) to give 0.006 g (0.009 mmol, 45%) of yellow oily solid. ^{1}H NMR (300 MHz, CDCl$_3$) δ 1.61-1.79 (m, 5 H), 1.80-2.04 (m, 8 H), 2.14-2.36 (m, 4 H), 2.98-3.14 (m, 2 H), 3.04 (s, 6 H), 3.25-.3.37 (app q, 2 H), 3.38-3.46 (m, 4 H), 3.52-3.68 (m, 12 H), 6.06 (s, 1 H), 6.49 (d, 1 H, J = 2.5), 6.60 (dd, 1 H, J = 2.5, 9.0), 6.62 (br t, 1 H), 7.13 (br t, 1H), 7.34 (d, 2 H, J= 7.9 hz), [7.40 (d, 0.6 H, J= 8.3 hz)], 7.74 (d, 2 H J= 8.4 hz), 7.51 (d, 1H, J= 9.0 hz), [8.03 (d, 0.6H, J=8.3 hz)]; FAB MS calculated for C$_{39}$H$_{51}$N$_3$O$_7$F (M+H) 692.3711, observed 692.3726.

3. Clooctyne acid **7** (0.0028 g, 0.014 mmol) was dissolved in 200 ml of CH$_2$Cl$_2$ in a foil wrapped flask and placed under argon. Triethylamine (8 µl, 0.057 mmol) was added, and the solution was cooled in an ice bath. Pentafluorophenyltrifluoroacetate (3 µl, 0.017 mmol) was added, and the solution was stirred for 1 h. The solvent was removed under reduced pressure, and the resulting oil was resuspended in 3 ml of 1:1 ethyl acetate: hexane and passed through a short plug of silica (in a pasteur

pipette). The solvent was removed under reduced pressure, and the material was resuspended in 1 ml of CH_2Cl_2. This solution was added to a solution of **10** (0.0125g, 0.023 mmol) that had been deprotected by treatment with 1.5 ml of 1:1 TFA:CH_2Cl_2 for 2 h, followed by solvent removal and azeotropic removal of TFA with toluene and resuspension in 0.2 ml CH_2Cl_2 and 30 µl (0.210 mmol) triethylamine. The combined solution was stirred under argon and monitored by TLC. After 3 h, the reaction mixture was applied directly to a silica column and eluted with 1:19 Ethanol:CH_2Cl_2 ($R_f = 0.22$) to give 5.3 mg (0.008 mmol, 64%) of a yellow glassy solid. ^{1}H NMR (300 MHz, CDCl$_3$) δ 1.68-1.87 (m, 4H), 1.98-2.08 (m, 2H), 2.13-2.31 (m, 3H), 2.39-2.63 (m, 3H), 3.06 (s, 6H), 3.31-3.45 (m, 4H), 3.45-3.69 (m, 15H), 3.92 (s, 2H), 6.07 (s, 1H), 6.48 (d, 1H J = 2.6), 6.61 (dd, 1H J = 2.9, 8.8), 6.79 (br t, 1H), 6.98 (br t, 1H), 7.51 (d, 2H, J = 9.3); FAB MS calculated for $C_{33}H_{46}N_3O_8F_2$ (M+H) 650.3253, observed 650.3233.
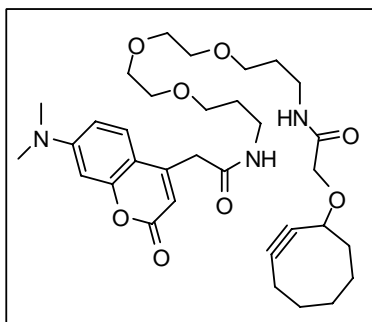
4. Clyclooctyne acid **8** (0.020 g, 0.110 mmol) was dissolved in 1ml of CH_2Cl_2 in a foil wrapped flask and placed under argon. Triethylamine (20 µl, 0.140 mmol) was added, and the solution was cooled in an ice bath. Pentafluorophenyltrifluoroacetate (20 µl, 0.116 mmol) was added, and the solution was stirred for 2 h. The solvent was removed under reduced pressure, and the resulting oil was resuspended in 3 ml of 1:1 ethyl acetate: hexane and passed through a short plug of silica (in a pasteur pipette). The solvent was removed under reduced pressure, and the material was resuspended in 1 ml of CH_2Cl_2. This solution was added to a solution of **10** (0.043g, 0.072 mmol) that had been deprotected by

treatment with 1:1 TFA:CH$_2$Cl$_2$ for 2 h, followed by solvent removal and resuspension in 2 ml CH$_2$Cl$_2$ and 22 µl (0.160 mmol) triethylamine. The combined solution was stirred under argon and monitored by TLC. After 4 h, the reaction mixture was applied directly to a silica column and eluted with 1:19 Ethanol:CH$_2$Cl$_2$ (R$_f$ = 0.22) to give 33 mg (0.054
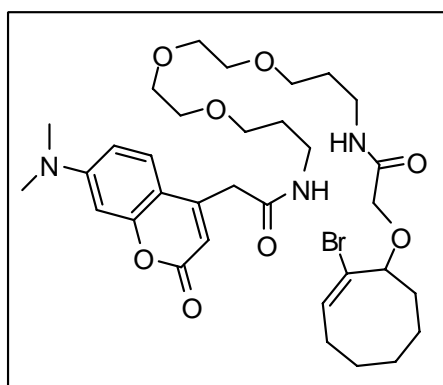
mmol, 99%) of a yellow glassy solid. This material was contaminated with ~30% of a byproduct that resulted from the coupling of bromocyclooctene acid that was carried through from the synthesis of the cyclooctyne acid. ^{1}H NMR (300 MHz, CDCl$_3$), with resonances in [] attributed to impurity: δ 0.87 (m, 1 H), 1.19-1.53 (m, 3 H), 1.54-2.06 (m, 8 H), 2.08-2.35 (m, 2 H), 3.06 (s, 6H), 3.31-3.42 (m, 4 H), 3.48-3.67 (m, 14 H), 3.80-3.90 (m, 1 H), 3.94-4.09 (m, 1 H), 4.20-4.28 (m, 1 H), [4.39 (dd, 0.3 H, J = 6.1, 9.8)], 6.07 (s, 1 H), [6.34 (t, 0.3 H, J = 8.9), 6.49 (d, 1 H, J = 2.5), 6.60 (dd, 1 H, J = 2.5, 9.0), 6.76 br t, 1 H, 6.84 br t, 1 H, [6.98 (br t, 0.3 H)],7.52 (d, 1 H, J = 8.7 hz); FAB MS calculated for C$_{33}$H$_{48}$O$_8$N$_3$ (M+H) 614.3441, observed 614.3428, [calculated for C$_{33}$H$_{49}$N$_3$O$_8$Br (M+H) 694.2703, obs 694.2749].

5.5.8   *In Vitro* Reactions and HPLC Analysis

Model reactions between 1 µM **3** and 1 mM amino acid were done in PBS (pH 7.4). Reactions proceeded at 37 °C until analysis. All liquid chromatography was on a Waters HPLC system with a Microsorb C18 column (Varian, Inc.) The buffers used for separation of the reaction components were 0.1% trifluoroacetic acid (Eluent A) and 100% Acetonitrile (Eluent B). The gradient was 0-2 min, 100% A; 2-4 min, 100-70% A;

4-14 min, 70-40% A; 14-28 min, 40-0% A. Dual detection at 280 and 350 nm was used to identify the substrate and product peaks, which were collected for analysis by electrospray ionization mass spectrometry at California Institute of Technology's Mass Spectrometry Facility [40].   The peak corresponding to unreacted **3** was confirmed [calculated $C_{33}H_{46}N_3O_8F_2$ (M+H) 650.3253, observed 650.1]. The peak corresponding to the Aha-product was collected and confirmed [calculated $C_{37}H_{53}F_2N_7O_{10}$ (M+H) 794.38, observed 794.25]. The peaks corresponding to the Cys-product were collected, but there was too little material to confirm the product(s). No product was observed for reactions of **3** with Met after 24 h.

The data were fitted to a single-exponential decay function using Origin software (OriginLab). The decrease in peak area (y) for **3** over time (t) was fitted to the equation:

$$y = y_o + Ae^{(-t/\tau)}$$

The time constant (τ) was converted to the half-life ($t_{1/2}$) using the equation:

$$t_{1/2} = \tau \ln(2)$$

Four experiments were averaged to obtain the half-life for Aha (16 ± 3 min) and Cys (27 ± 5 min).

Further work is underway to identify the products observed by HPLC and to examine model reactions of Aha, Cys, and Met with coumarins **1** and **2**.

5.6   Acknowledgements

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