

Chapter 3

Presentation and Detection of Azide Functionality in Bacterial Cell Surface Proteins

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Abstract

An improved protocol for copper-catalyzed triazole formation on the bacterial cell surface is described. Addition of highly-pure CuBr to cells treated with azidohomoalanine (**2**) leads to ca. 10-fold more extensive cell surface labeling than previously observed. This highly active catalyst allows detection of the methionine analogues azidoalanine (**1**), azidonorvaline (**3**), and azidonorleucine (**4**) in cell-surface proteins. Azidoalanine was previously believed to be silent with regard to the cellular protein synthesis machinery.

Introduction

The utility of non-canonical amino acids in protein engineering has become apparent in recent years,¹ as striking examples of increased protein stability^{2,3} and large spectral shifts in fluorescent proteins⁴ have been effected by replacement of natural amino acids with non-canonical analogs. Another important technology enabled by non-canonical amino acids is the ability to introduce novel chemical functionality into proteins and cells. Among the functional groups incorporated in this way are the acetyl group,^{5,6} the benzophenone moiety,⁷ halogens,⁸ and azides.⁹

Generally, the incorporation of non-canonical amino acids is detected by mass spectrometry of either the intact protein or its proteolytic fragments. Other methods of analysis include immunoblotting,⁹ amino acid analysis, N-terminal sequencing,¹⁰ or NMR.¹¹ We recently reported an alternate strategy for the detection of non-canonical amino acids, in which azidohomoalanine (**2**, Figure 1) was incorporated into the *Escherichia coli* outer membrane protein OmpC.¹² The azides on the cell surface can be covalently biotinylated via copper-catalyzed triazole formation and subsequently stained with fluorescent streptavidin, facilitating flow cytometric separation of labeled and unlabeled cells. We show here that by combining this cell-surface display strategy with a novel, more efficient copper source, we are able to detect incorporation of azidoalanine (**1**), an analogue of methionine previously thought to be translationally silent. We also demonstrate incorporation of the larger amino acids azidonorvaline (**3**) and azidonorleucine (**4**) in place of methionine.

Experimental Section

Synthesis of Azido Amino Acids 1-4 and Biotin-PEO-propargylamide 6. All chemicals were purchased from commercial suppliers and used without further purification. Mass spectrometric analyses were performed either at the Caltech mass spectrometry facility or at the mass spectrometry facility at the University of Amsterdam (Swammerdam Institute for Life Sciences).

Azidoalanine (**1**) was synthesized by diazo transfer¹³ to commercially available *N*- α -Boc-protected diaminopropionic acid (Boc-Dap-OH, Bachem Bioscience Inc., King of Prussia, PA, USA). Triflic anhydride (Tf₂O, 3.00 mL, 17.8 mmol) was added dropwise to a vigorously stirred mixture of NaN₃ (5.76 g, 88.6 mmol) in H₂O (15 mL) and CH₂Cl₂ (30 mL) at 0 °C. The resulting mixture was allowed to warm to rt and stirred for 2 h. The water layer was extracted with CH₂Cl₂ (2 × 15 mL) and the combined organic layers were washed with saturated aqueous Na₂CO₃ (25 mL). The resulting solution of TfN₃ in CH₂Cl₂ was subsequently added slowly to a solution of Boc-Dap-OH (1.81 g, 8.86 mmol), K₂CO₃ (1.84 g, 13.3 mmol) and CuSO₄·5H₂O (22 mg, 0.088 mmol) in H₂O (30 mL), and MeOH (45 mL). The resulting mixture was stirred overnight, and the organic solvents were evaporated under reduced pressure. The water layer was acidified to pH 6 with conc HCl, diluted with 0.25 M pH 6.2 phosphate buffer (50 mL), and extracted with CH₂Cl₂ (4 × 100 mL). The combined organic layers were washed with brine (50 mL), dried over Na₂SO₄, and concentrated *in vacuo*. The residue was dissolved in 5 mL conc HCl (5 mL), and after stirring for 24 h, the product was purified using cationic Dowex exchange resin to afford the product as an off-white powder (696 mg, 60%). ¹H NMR

(300 MHz, D₂O): 3.80-3.63 (m, 3H). ¹³C NMR (75 MHz, D₂O): 171.8, 53.9, 50.8. HRMS (FAB) calculated for C₃H₇N₄O₂ (MH⁺) 131.0569, found 131.0569.

2-Amino-5-azidopentanoic acid (azidonorvaline, **3**): Boc-Ornithine-Z (Boc-Orn(Z)-OH, 2.00 g, 5.46 mmol, Novabiochem) and 10% Pd/C (581 mg, 0.819 mmol Pd) in EtOAc (50 mL) were subjected to a hydrogen atmosphere of 40 psi for 16 h. The mixture was filtered over Celite[®], the filter was washed with water, and the solvents were evaporated *in vacuo*. The resulting product (1.7 g) was used in the next reaction without further purification. Tf₂O (1.84 mL, 10.9 mmol) was added dropwise to a vigorously stirred mixture of NaN₃ (3.55 g, 54.6 mmol) in H₂O (9 mL) and CH₂Cl₂ (15 mL) at 0 °C. The resulting mixture was allowed to warm to rt and stirred for 2 h. The water layer was extracted with CH₂Cl₂ (2 × 5 mL), and the combined organic layers were washed with saturated aqueous Na₂CO₃ (25 mL). The resulting solution of TfN₃ in CH₂Cl₂ was subsequently added slowly to a solution of Boc-ornithine, K₂CO₃ (1.13 g, 8.18 mmol) and CuSO₄·5H₂O (14 mg, 0.056 mmol) in H₂O (17 mL), and MeOH (34 mL). The mixture was stirred overnight, and the organic solvents were evaporated under reduced pressure. The water layer was acidified to pH 6 with conc HCl, diluted with 0.25 M pH 6.2 phosphate buffer (50 mL), and extracted with CH₂Cl₂ (4 × 100 mL). The combined organic layers were washed with brine (50 mL), dried over Na₂SO₄, and concentrated *in vacuo*. The residue was dissolved in conc HCl (5 mL), and after stirring for 24 h, the product was purified using cationic Dowex exchange resin to afford the product as an off-white powder (382 mg, 41%). ¹H NMR (600 MHz, D₂O): 3.70 (t, J = 6.1 Hz, 1H), 3.34 (t, J = 6.6 Hz, 2H), 1.92-1.84 (m, 2H), 1.69-1.56 (m, 2H). ¹³C NMR (75 MHz, D₂O):

174.9, 54.6, 50.7, 28.1, 24.2. HRMS (EI) calculated for $C_5H_{10}N_4O_2$ 158.0804, found 158.0170.

2-Amino-6-azidohexanoic acid (azidonorleucine, **4**): Tf_2O (2.7 mL, 16 mmol) was added dropwise to a vigorously stirred mixture of NaN_3 (5.27 g, 81.1 mmol) in H_2O (13 mL) and CH_2Cl_2 (22 mL) at 0 °C. The resulting mixture was allowed to warm to rt and stirred for 2 h. The water layer was extracted with CH_2Cl_2 (2×8 mL), and the combined organic layers were washed with saturated aqueous Na_2CO_3 (25 mL). The resulting solution of TfN_3 in CH_2Cl_2 was subsequently added slowly to a solution of *N*- α -Boc-lysine (2.0 g, 8.1 mmol, Bachem), K_2CO_3 (1.68 g, 12.2 mmol) and $CuSO_4 \cdot 5H_2O$ (20 mg, 0.080 mmol) in H_2O (26 mL), and MeOH (52 mL). The mixture was stirred overnight, and the organic solvents were evaporated under reduced pressure. The water layer was acidified to pH 6 with conc HCl, diluted with 0.25 M pH 6.2 phosphate buffer (50 mL), and extracted with CH_2Cl_2 (4×100 mL). The combined organic layers were washed with brine (50 mL), dried over Na_2SO_4 , and concentrated *in vacuo*. The residue was dissolved in conc HCl (5 mL), and after stirring for 24 h, the product was purified using cationic Dowex exchange resin to afford the product as a white powder (244 mg, 18%). 1H NMR (600 MHz, D_2O): 3.69-3.67 (m, 1H), 3.30 (t, $J = 6.6$ Hz, 2H), 1.86-1.78 (m, 2H), 1.62-1.57 (m, 2H), 1.44-1.35 (m, 2H). ^{13}C NMR (75 MHz, D_2O): 177.6, 57.4, 53.4, 32.7, 30.4, 24.4. HRMS (FAB) calculated for $C_6H_{13}N_4O_2$ (MH^+) 173.1039, found 173.1040.

Azidohomoalanine (**2**) was prepared as previously described.¹⁴ Biotin-PEO-propargylamide (**6**) was synthesized by reacting an activated biotin-PEO ester with excess neat propargylamine as described elsewhere.¹²

Plasmids and Expression Hosts. The plasmid pQE-60/OmpC encodes a mutant form of outer membrane protein C that contains six additional methionine residues in its exposed loops.¹² pQE-60/OmpC was linearized by digestion with *Nhe* I. The plasmid pQE-15 MRS, which encodes a methionyl-tRNA synthetase expression cassette¹⁵ was digested with *Nhe* I, and a 2.5 kb fragment corresponding to the MetRS cassette was isolated by agarose gel electrophoresis. This fragment was ligated to the linearized pQE-60/OmpC to generate the plasmid pAJL-20. This plasmid was transformed into the *E. coli* methionine auxotroph M15MA¹² to generate the expression host M15MA[pAJL-20]. The expression host M15MA[pQE-60/OmpC] has been previously described.¹²

Expression of OmpC Containing Non-Canonical Amino Acids. An overnight culture of the expression host was diluted 50-fold in M9 minimal medium containing all twenty natural amino acids (40 mg/L each) as well as the antibiotics ampicillin (200 mg/L) and kanamycin (35 mg/L). Upon reaching an OD₆₀₀ of 0.9-1, the culture was centrifuged at 6000 g for 7 min and resuspended in M9 medium containing 19 amino acids (no methionine). The cells were shaken at 37 °C for ten minutes to deplete the intracellular pool of methionine, centrifuged again, and resuspended in M9 medium containing 19 amino acids (no methionine). To these cells was added either methionine, the non-canonical amino acid, or no amino acid. Methionine (**7**) and azidohomoalanine (**2**) were added to a final concentration of 40 mg/L while **1**, **3**, and **4** were added to a final concentration of 750 mg/L. Protein expression was induced for three hours at 37 °C by the addition of isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 1 mM.

Copper-Catalyzed Triazole Formation on the Cell Surface. An aliquot of cells expressing recombinant OmpC (1 mL) was centrifuged at 4 °C and washed once in 1 mL of PBS (pH 7.4). The cells were centrifuged and resuspended in 1 mL of PBS. Triazole ligand **5** was added to a final concentration of 200 μ M and biotin-PEO-propargylamide **6** was added to a final concentration of 50 μ M. Addition of the active copper species was accomplished in two different ways. For *in situ* generation of Cu(I), 100 μ M CuSO₄ and 200 μ M of tris(carboxyethyl)phosphine (TCEP) were added to the cells. Alternatively, the Cu(I) ion was added directly to the cells in the form of an aqueous suspension of CuBr. Briefly, 10 μ L of a 10 mM suspension of CuBr (99.999% purity, Aldrich) was thoroughly agitated and added to the cells. As discussed in the Results section, the quality of the CuBr is critical for the success of the experiment. All labeling reactions were allowed to continue for 16 h at 4 °C and were stopped by washing the cells with PBS.

Outer Membrane Fraction Isolation and Western Blotting. The outer membrane protein fraction of cells labeled via the azide-alkyne cycloaddition was isolated as described.¹⁶ The outer membrane protein fractions from equal numbers of cells were electrophoresed (12 % tris-tricine gel, 165 V, 45 min) and subsequently transferred to a nitrocellulose membrane (30 V, 1 hr at 4 °C). The membrane was blocked for 1 hr with 5 % milk in PBS/Tween. After washing with PBS, the membrane was probed with an avidin-HRP conjugate (Amersham Biosciences). Blots were visualized using appropriate detection reagents (ECL Western Blotting Analysis System, Amersham Biosciences).

Flow Cytometry. After OmpC expression and biotinylation, 1 mL of cells was washed twice with 1 mL of PBS (pH 7.4). The cells were stained with 5 μ L of a 1 mg/mL solution of an avidin-Alexa Fluor 488 conjugate (Molecular Probes, Eugene, OR) for 2-3 h at 4 °C. The cells were washed three more times with PBS, diluted 40-fold, and filtered through a 5 μ m syringe filter before flow cytometry. A MoFlo cell sorter (DakoCytomation, Ft. Collins, CO) was used for cytometric analysis. At least 20,000 events were collected in each experiment. Data analysis was performed with Summit software (DakoCytomation).

Results and Discussion

Copper-Catalyzed Triazole Formation on the Surfaces of Intact Cells. There are several examples of bioconjugation via copper-catalyzed triazole formation (Scheme 1) in the literature to date.¹⁷⁻¹⁹ These conjugation strategies all rely on catalysis by Cu(I).²⁰ In all of these experiments, as well as in our previous work, the Cu(I) species was generated *in situ* from the soluble Cu(II) source CuSO₄ and an exogenous reducing agent, either a copper wire or TCEP. Direct addition of Cu(I) salts to proteins or cells to effect bioconjugation has likely been avoided because of the extremely low solubility of these salts in aqueous media and their tendency to disproportionate quickly to Cu(0) and Cu(II). Despite these reservations, we decided to investigate the efficiency of Cu(I) salts in effecting triazole formation on the surface of *E. coli*.

Cells displaying azidohomoalanine (**2**) on the cell surface were generated by inducing expression of the outer membrane protein OmpC in medium supplemented with

40 mg/L of **2**. The expression vector in these experiments, pAJL-20, contains an extra copy of the gene for methionyl-tRNA synthetase (MetRS), though nearly identical results are observed with the expression vector pQE-60/OmpC, which lacks the extra copy of MetRS. Given that overexpression of MetRS is not required for the incorporation of **2** into proteins,^{9,12} it is expected that similar amounts of OmpC containing **2** will be produced in expression strains harboring either pAJL-20 or pQE-60/OmpC. Following protein expression, the cells were labeled by triazole synthesis with either CuSO₄/TCEP or CuBr as the copper source. Following isolation of the outer membrane fractions of these cells and Western blotting, it is clear that CuBr leads to a much larger extent of cell surface labeling (Figure 2). As in our previous experiments, the labeling reaction is highly specific to cells displaying azides. When cells are induced in media supplemented either with methionine or no analogue and subjected to triazole synthesis reaction conditions, no appreciable biotinylation is observed (see Figure 2). Use of CuBr as the copper source also yields evidence of incorporation of azidohomoalanine into outer membrane proteins other than OmpC. Some level of incorporation into proteins other than OmpC is expected since protein synthesis is not halted completely upon induction of OmpC; nevertheless, this low level of incorporation into other cellular proteins was never observed when CuSO₄/TCEP was used as the Cu(I) source.¹² It should be noted that the purity of the CuBr is integral to the success of the experiment. The results described above were obtained only with a 99.999% pure sample stored under dry conditions. Use of a 98% pure preparation of CuBr leads to only modest extents of reaction as evidenced by Western blotting (data not shown).

The increased extent of cell surface labeling is more strikingly demonstrated in a flow cytometry experiment. Following biotinylation, cells were stained with fluorescent avidin and analyzed by flow cytometry. Cells treated with CuSO₄ and TCEP exhibit a median fluorescence intensity about 20-fold higher than the background fluorescence of *E. coli*. When CuBr is used as the copper source, the median fluorescence of labeled cells is 150-fold higher than the background (Figure 3). The high specificity of labeling is also evident in flow cytometry experiments. When cells induced in media supplemented with either methionine or no analogue were subjected to biotinylation with CuBr and subsequent staining with fluorescent streptavidin, only background levels of fluorescence were observed. Use of CuBr leads to excellent baseline separation between the labeled and unlabeled populations; the labeled population is easily sorted to > 98% purity.

Incorporation of Azidoalanine. Our previous investigation of azidoalanine (**1**) led to the conclusion that azidoalanine is not an efficient surrogate for methionine in protein synthesis.⁹ This determination was made on the basis of two kinds of evidence. First, no protein expression was seen in SDS-PAGE analyses of lysates of cells induced in media supplemented with **1**. Furthermore, **1** does not support any perceptible ATP exchange in an *in vitro* assay of methionyl-tRNA synthetase activity. Nonetheless, when cells transformed with pAJL-20 were induced in medium supplemented with 750 mg/L (5.76 mM) of **1** and labeled with CuBr as the copper source, azidoalanine incorporation into OmpC was readily detected. The extent of labeling of cells by reaction of **1** is significantly lower than that characteristic of **2** as evidenced by Western blotting of outer

membrane fractions (Figure 4). In the corresponding flow cytometry experiment, cells incorporating azidoalanine exhibit a median fluorescence intensity approximately 10-fold higher than unlabeled cells (Figure 5). The use of CuBr as a catalyst is again critical for successful detection of azidoalanine incorporation. When CuSO₄/TCEP was used as the Cu(I) source, only background fluorescence intensities were observed in the flow cytometry experiment (data not shown).

Incorporation of Azidonorvaline and Azidonorleucine. We next turned our attention to the incorporation of the larger azido amino acids azidonorvaline (**3**) and azidonorleucine (**4**). The side chains of **3** and **4** are both significantly longer than that of methionine. Nevertheless, when M15MA[pAJL-20] cells were induced in media containing of 750 mg/L of either **3** or **4** and biotinylated via the triazole synthesis reaction, incorporation of both amino acids was observed by Western blotting (Figure 6). As with azidoalanine, the extent of labeling of cells treated with **3** or **4** is significantly lower than that of cells treated with azidohomoalanine. Incorporation of **3** and **4** was also observed in flow cytometry experiments (Figure 7). Following biotinylation and staining with fluorescent streptavidin, cells induced in media supplemented with **3** exhibited a fifteen-fold increase in median fluorescence over the background, while cells induced in media supplemented with **4** exhibited a twenty-fold increase. When similar experiments were performed in media supplemented with 40 mg/L methionine, no incorporation of either **3** or **4** was detected by Western blotting or flow cytometry, supporting the hypothesis that **3** and **4** act as methionine surrogates. Finally, as was the case with azidoalanine, neither **3** nor **4** supported detectable ATP-PP_i exchange in *in vitro* assays of

methionyl-tRNA synthetase activity. It should also be noted that when M15MA[pQE-60/OmpC] is used as the expression host, only background fluorescence intensities are observed in flow cytometry experiments with **1**, **3**, and **4**, demonstrating the importance of MetRS overexpression for the incorporation of these analogues.

Conclusions

The expression of an outer membrane protein in medium supplemented with a non-canonical amino acid provides a sensitive means of detecting analogue incorporation. The strategy is dependent on the chemistry used to label the amino acid following its incorporation into the outer membrane protein. By using a more efficient catalyst in the current study, we have been able to increase the extent of surface labeling of cells incorporating **2** nearly 10-fold in comparison with previous work. The high efficiency of labeling observed in this study indicates the extent of labeling in our previous work (using CuSO₄/TCEP) could not have exceeded 10-15% of all possible azide sites of the cell surface. Highly efficient labeling, such as that afforded by the CuBr catalyst, may be necessary to detect inefficient incorporation of reactive amino acids. This assertion is borne out by the fact that incorporation of **1** was detected in the current study only when the CuBr catalyst was used. Use of the CuBr catalyst also allows detection of the incorporation of the new methionine analogues **3** and **4**. It is particularly surprising that these amino acids serve as methionine surrogates given their large size.

It is important to stress that the cell-surface display strategy described herein need not be limited to detection of azide-bearing amino acids only; any labeling chemistry that

can be carried out in aqueous media at physiological temperatures should be useful in detecting non-canonical amino acids on the cell surface.²¹ Furthermore, flow cytometry also allows sorting of labeled and unlabeled cells and is therefore well-suited for screening libraries of cells for mutant strains that can use non-natural amino acids in protein synthesis.

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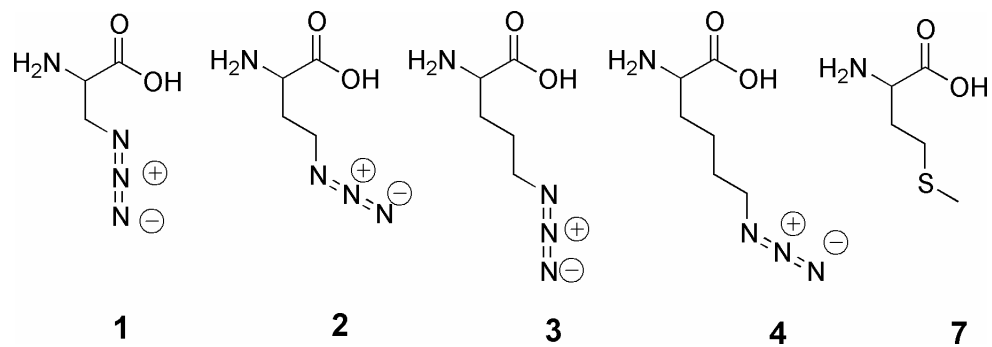
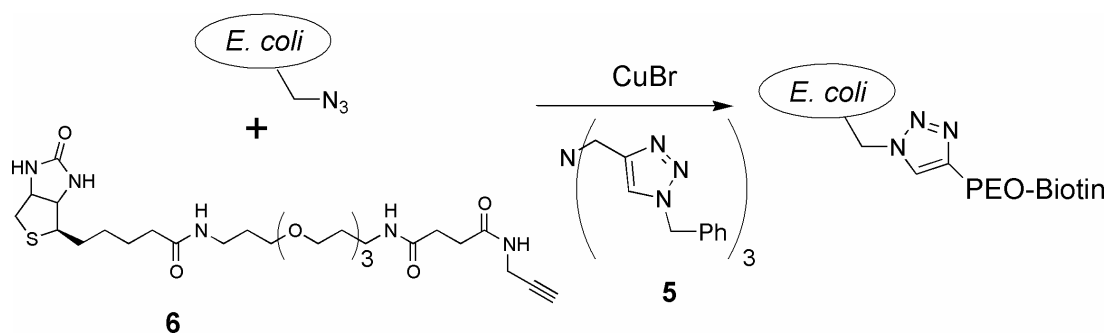


Figure 1: Amino acids discussed in the current study. **1:** azidoalanine, **2:** azidohomoalanine, **3:** azidonorvaline, **4:** azidonorleucine, **7:** methionine.



Scheme 1: Copper-catalyzed triazole formation on the *E. coli* cell surface. Metabolically incorporated azides on the cell surface are reacted regiospecifically with biotin-PEO-propargylamide **6** in the presence of Cu(I) and triazole ligand **5**.

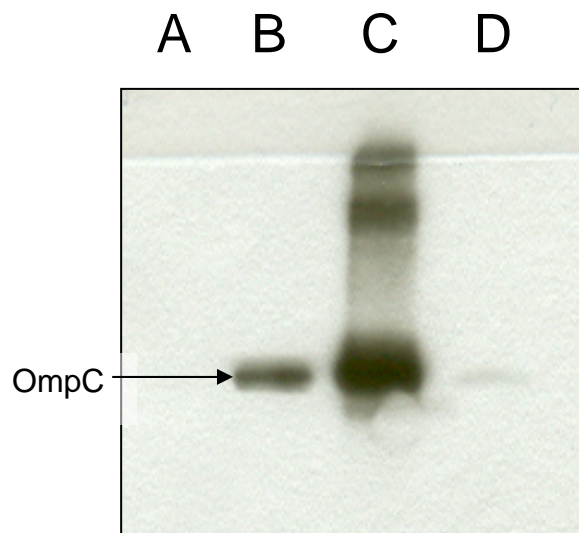


Figure 2: Comparison of extent of labeling of cell surface proteins in triazole syntheses catalyzed by different copper sources. Lane A: Outer membrane proteins (OMP) from cells induced in medium supplemented with methionine and subjected to triazole synthesis reaction conditions with CuBr as Cu(I) source. Lane B: OMP from cells induced in media supplemented with **2** and labeled with CuSO₄/TCEP as the Cu(I) source. Lane C: Same as B, except CuBr as Cu(I) source. Lane D: OMP from cells induced in media containing 19 amino acids (no methionine) and subjected to triazole reaction conditions with CuBr. Note labeling of proteins other than OmpC when CuBr is used (lane C).

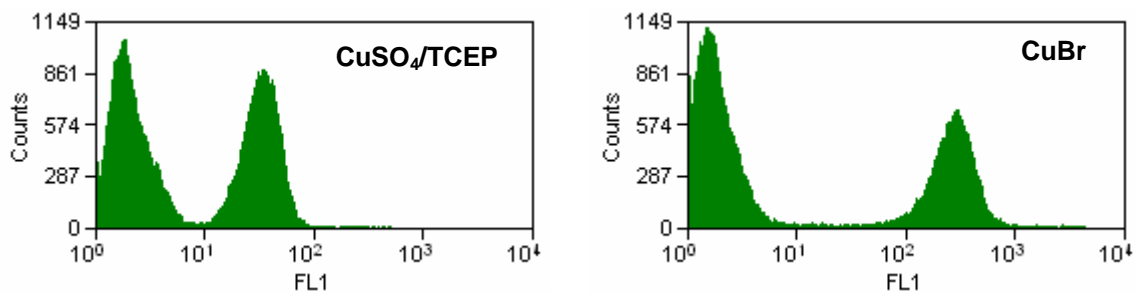


Figure 3: Fluorescence histograms of mixtures of cells induced in media supplemented with 19 amino acids (no methionine) and cells incorporating **2** and labeled by copper-catalyzed triazole formation. Following induction, cells were subjected to biotinylation reaction conditions, stained with fluorescent avidin, and mixed just prior to flow cytometric analysis. As mentioned in the text, cells induced in the presence of no methionine have only background levels of fluorescence. Cells incorporating **2** and labeled with $\text{CuSO}_4/\text{TCEP}$ as a copper source exhibit median fluorescence intensity 20-fold higher than background while cells labeled in the presence of CuBr exhibit a 150-fold increase.

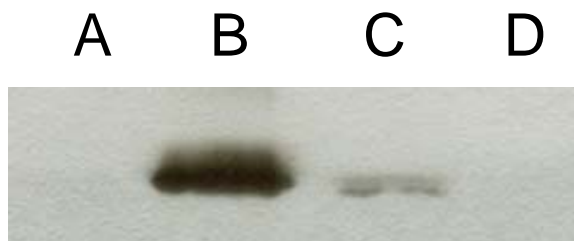


Figure 4: Comparison of extent of labeling of cells incorporating either **1** or **2**. Lane A: OMP from cells induced in the presence of 40 mg/L methionine. Lane B: OMP from cells induced in the presence of 40 mg/L **2**. Lane C: OMP from cells induced in the presence of 750 mg/L **1**. Lane D: OMP from cells induced in the presence of 19 amino acids (no methionine). CuBr was used as the copper source in all lanes.

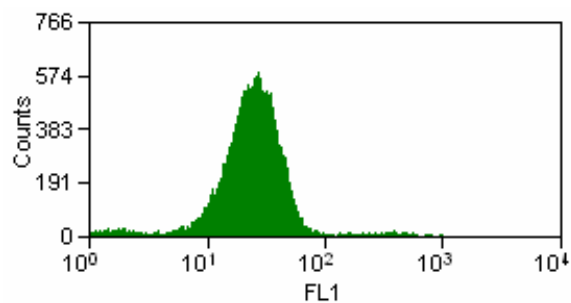


Figure 5: Detection of azidoalanine incorporation by flow cytometry. Cells were induced in media supplemented with 750 mg/L azidoalanine, subjected to triazole synthesis reaction conditions, and stained with fluorescent avidin. Cells incorporating azidoalanine have median fluorescence intensity 10-fold greater than the background.

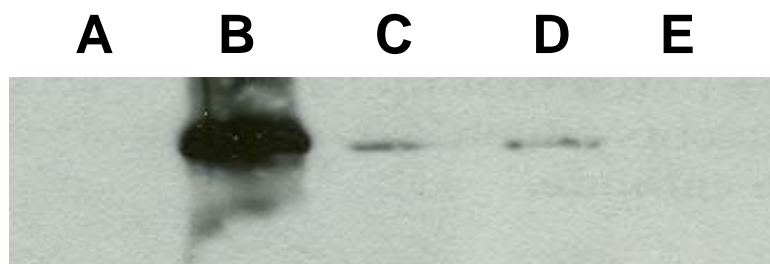


Figure 6: Western blot of OMPs prepared from cells induced in media supplemented with **2**, **3**, or **4** and biotinylated via triazole synthesis. Media were supplemented with A: 40 mg/L methionine, B: 40 mg/L **2**, C: 750 mg/L **3**, D: 750 mg/L **4**, E: no amino acid.

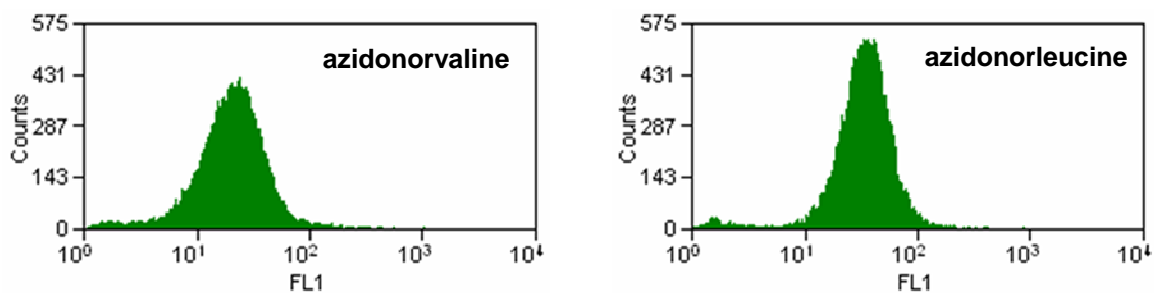


Figure 7: Fluorescence histograms of cells induced in media supplemented with either azidonorvaline (**3**) or azidonorleucine (**4**) at 750 mg/L, biotinylated via triazole synthesis, and stained with fluorescent avidin. Cells treated with **3** exhibit a 15-fold increase in fluorescence over background while cells treated with **4** exhibit a 20-fold increase.