

Global Incorporation of Unnatural Amino Acids in *Escherichia coli*

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Summary

The incorporation of amino acid analogs is becoming increasingly useful. Site-specific incorporation of unnatural amino acids allows the application of chemical biology to protein-specific investigations and applications. However, the global incorporation of unnatural amino acids allows for tests of proteomic and genetic code hypotheses. For example, the adaptation of organisms to unnatural amino acids may lead to new genetic codes. To understand and quantify changes from such perturbations, an understanding is required of the microbiological and proteomic responses to the incorporation of unnatural amino acids. Here we describe protocols to characterize the effects of such proteome-wide perturbations.

Key Words: Unnatural amino acids; genetic code ambiguity; amino acid misincorporation; amino acylation errors; amino acid analogs; genetic code evolution.

1. Introduction

The overexpression of proteins that contain unnatural amino acids is becoming increasingly interesting for a number of reasons. As one example, the site-specific incorporation of unnatural amino acids allows specific tests of chemical hypotheses regarding protein structure and function. Similarly, the site-specific incorporation of unnatural amino acids with novel chemistries may foment new protein functions, such as crosslinking with keto-substituted amino acids (1). Global incorporation of unnatural amino acids throughout a protein can also lead to novel physical or functional properties. For example, the global replacement of methionine with heavy-atom analogs, such as selenomethionine, has become a useful tool for obtaining difference maps in X-ray crystallography (2). Global perturbation of organismal proteomes with unnatural amino acids has also been used as a means of experimentally probing the evolution of

the genetic code. Both bacteria and phage have been adapted to incorporate unnatural amino acids, and the number and type of mutations that were required for these evolutionary transitions have been examined (3–6).

Although there is a body of literature regarding the forced growth of bacteria on unnatural amino acids and the subsequent isolation of proteins containing unnatural amino acids (for examples using tryptophan analogs, *see refs. 7–11*), for the most part, these are just technical descriptions; there is no consideration of how changes in protocol will affect the outcome of these experiments. We, therefore, present a more-detailed account of methods for whole-protein or whole-cell incorporation of unnatural amino acids, with special emphasis on the incorporation of tryptophan analogs.

2. Materials

1. *E. coli* strains C600 Δ *trpE* (*thi-1 thr-1 leuB6 lacY1 tonA21 supE44 mcrA* Δ *trpE*) and derivative strains, C600p (C600 Δ *trpE* + pUC18), C600pGSR (C600 Δ *trpE* + pGSR), C600F (C600 Δ *trpE* F'KanR), and C600F(DE3) (C600F λ DE3 lysogen). Strains used for transformation include DH5 α F' and TOP10 (Invitrogen, Carlsbad, CA).
2. Luria-Bertani media (per liter: 10 g tryptone, 5 g yeast extract, 10 g NaCl, and 1.5% bacto-agar for plates) and minimal media M9 (5X stock solution, per liter: 30 g Na₂HPO₄, 15 g KH₂PO₄, 5 g NH₄Cl, 2.5 g NaCl, and 1.5% bacto-agar for plates), supplemented with 20 μ g/mL amino acids (*see Note 1*) and 0.0005% thiamine. Rich and minimal media are supplemented with antibiotics, 50 μ g/mL ampicillin (Ap) or kanamycin (Kn), as indicated.
3. Tryptophan analogs: 4-, 5-, and 6-fluorotryptophan (fW), Sigma (St. Louis, MO).
4. Plasmids: pGEX-KG (12), pET100/D-topo (Invitrogen), for high-level expression of proteins. For polymerase chain reaction (PCR) amplification of genes, pGFPuv (Clontech, La Jolla, CA) or another source of the gene encoding GFPuv and a plasmid source of the Kn kinase gene (such as p182Sfi-, Kan, K. A. Marshall and A. D. Ellington, unpublished).
5. Vent and Taq DNA polymerases, restriction endonucleases, DNase, T4 kinase, and T4 DNA ligase.
6. Oligonucleotide primers and dNTPs (Invitrogen).
7. Bacterial protein extraction reagent (B-PER) and B-PER II (Pierce, Beverly, MA).
8. 100 mM stock solution of isopropyl- β -D-thiogalactopyranoside (IPTG) in water.
9. Microcon concentrators, 10,000 molecular weight cutoff (Microcon, Rockford, IL).
10. Glutathione-Sepharose beads (Amersham Biosciences, Piscataway, NJ).
11. Ni-nitrilotriacetic acid (NTA) resin (Novagen), and protein purification columns (Bio-Rad, Hercules, CA).
12. Centri-Sep size-exclusion columns (Princeton Separations, Adelphia, NJ).
13. L-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Pierce, Beverly, MA).

14. Phosphate-buffered saline (PBS): 10X stock solution, per liter: 80 g NaCl, 2 g KCl, 11.5 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, and 2 g KH_2PO_4 .
15. 1 M MgCl_2 .
16. 50 mM Tris-HCl, pH 8.0, and 5 mM reduced glutathione.
17. Buffers for Ni-NTA purification:
 - a. Binding buffer, 8X stock: 160 mM Tris-HCl, pH 7.9, 4 M NaCl, and 40 mM imidazole.
 - b. Wash buffer, 8X stock: 160 mM Tris-HCl, pH 7.9, 4 M NaCl, and 480 mM imidazole.
 - c. Elution buffer, 8X stock: 160 mM Tris-HCl, pH 7.9, 4 M NaCl, and 2 M imidazole.
18. Buffers for high-performance liquid chromatography (HPLC)–HPLC analysis:
 - a. Buffer A: 50 mM NH_4OAc , pH 5.0.
 - b. Buffer B: 50 mM NH_4OAc , pH 5.0 and 50% MeOH.
 - c. Buffer C: 0.1 M NaH_2PO_4 , pH 2.5.
 - d. Buffer D: 0.1 M NaH_2PO_4 , pH 2.5 and 50% MeOH.
19. Agarose DNA gel equipment and sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE) equipment.
20. HPLC, HPLC-electrospray ionization (ESI), and mass spectrometry equipment.
21. Microplate reader.

3. Methods

The methods described below outline:

1. The growth of *E. coli* on tryptophan analogs.
2. The expression and purification of proteins with fW-substituted amino acids.
3. Methods for the analysis of levels of incorporation of unnatural amino acids.

3.1. Growth of *E. coli* on Unnatural Amino Acids

Incorporation of tryptophan analogs into *E. coli* is straightforward. Because tryptophanyl-transfer RNA synthetase has no editing domain, discrimination between the natural amino acid and analogs is based solely on structural determinants. Fluorinated tryptophan analogs are charged relatively effectively by the *Bacillus subtilis* tryptophanyl-transfer RNA synthetase; 4fW is charged six-fold less efficiently than W and 5fW is charged 74-fold less efficiently than W (13). Similarly, tryptophan analogs are known to enter the cell and support growth, for at least a few generations, before toxicity of the analog takes effect. To achieve efficient incorporation of tryptophan analogs, bacterial strains with mutations preventing tryptophan biosynthesis are used (see Note 2; refs. 4–6, 9, and 11). In the examples presented below, the strain used was *E. coli* C600 $\Delta trpE$, and derivatives. Minimal media supplemented with threonine, leucine, and thiamine, as well as tryptophan, analog, or some ratio of unnatural to natural amino acid. By convention, media is named by its supplements.

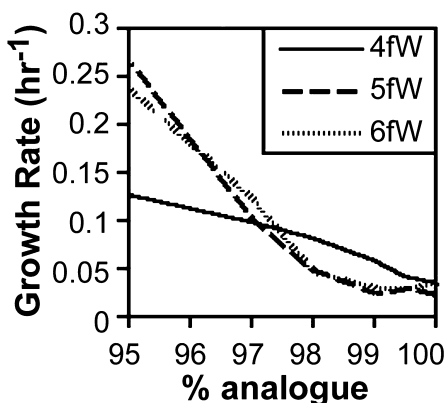


Fig. 1. Effect of various ratios of analog to natural tryptophan on the growth of *E. coli*. Strain C600p was grown on various ratios of 4fW (—), 5fW (---), and 6fW (.....). Growth was followed by spectrophotometry in microplates, and rates were determined using **Eq. 1**. (Data is reprinted with permission from **ref. 5**, but reanalyzed according to this equation.)

M9B1TL95% 4fW+Ap, for example, indicates minimal M9 media supplemented with vitamin B1 (thiamine), threonine, leucine, 19:1 4fW:W, and Ap.

The effect of unnatural amino acid incorporation on the growth capabilities of bacteria can be determined by analyzing growth curves. Instruments such as the ELX808 Microplate Reader (**14**) or the Bioscreen C (**5**) can take multiple growth curves in parallel in a microplate format. For testing in the Bioscreen C, an overnight culture of C600p in minimal permissive medium was diluted 1:100 to inoculate 1.5 mL of each medium to be tested for growth inhibition. Of these 1.5 mL media, 350 μ L was aliquoted into triplicate wells. Growth was tested with constant shaking at 37°C until all cultures reached the stationary phase. The growth rate was then calculated by fitting the logarithmic portion of the growth curve to the equation:

$$N_{(t)} = N_{(0)} \times e^{(-rt)} \quad (1)$$

where t = time, $N_{(t)}$ = the population (or optical density) at time t , and $N_{(0)}$ is the initial population density, and solving for r , which is the intrinsic growth rate. For example, fitting the exponential portion of growth to **Eq. 1** showed that 4fW causes little change in growth rate at ratios less than 97% relative to W, whereas the effects of 5fW and 6fW are more dramatic (**Fig. 1**). As such, for routine growth of bacteria, a ratio of 19:1 4fW:W can be used in the media. Growth of *E. coli* can be continued for several generations if only 4fW is supplied in the media. In addition, a more complete understanding of the growth curve can be

achieved by fitting the entire curve (as opposed to only the logarithmic phase) to the logistic growth equation, which takes into account the carrying capacity:

$$N_{(t)} = \frac{K}{1 + \{(K/N_{(0)}) - 1\} \times e^{(-\mu t)}} \quad (2)$$

where variables are the same as in **Eq. 1**, with the addition of K , which is the carrying capacity of the culture. This equation has been used to quantify the effect of amino acid analog incorporation in *E. coli* under conditions of amino acylation errors (**15**).

3.2. Expression and Purification of Proteins With 4fW Incorporation

This section describes the steps taken to construct two expression plasmids (**Subheadings 3.2.1.1.** and **3.2.1.2.**), and to express and purify the proteins under conditions of high substitution of W by 4fW (**Subheadings 3.2.2.1.** and **3.2.2.2.**). In terms of expression and purification, the protocol adaptations required for growth on amino acid analogs are slight relative to growth only on natural amino acids. Finally, this section will outline a method to isolate total cellular protein from bacteria that have incorporated unnatural amino acids (**Subheading 3.2.3.**).

3.2.1. Expression Vector Construction

3.2.1.1. pGSR

Because of the pUC18 plasmid in C600p, pGEX-KG, a glutathione-*S*-transferase (GST) expression vector, required a form of selection other than Ap. Therefore, the plasmid was digested with *Sma*I and *Eco*RI. The *Kn* kinase gene of p182Sfi-Kan was amplified via PCR using the primers Kan1.39 (5'-CGCG-GATCCGGCCACCATGGCCAAGCGAACCGGAAT) and Kan2.39 (5'-CCG-GAATTCTGAGGCCTGACAGGCCTTAGAAGAAGACTCGT). The PCR product was digested with *Bsa*BI and *Eco*RI and ligated into *Sma*I- and *Eco*RI-digested pGEX-KG (**16**). The ligation was transformed into DH5 α F', and isolated by miniprep (QIAGEN). The resulting plasmid, pGSR, is a GST expression vector that confers *Kn* resistance.

3.2.1.2. PET100GFPuv

The gene for the highly fluorescent GFPuv was amplified via PCR from the plasmid pGFPuv (Clontech) using Vent DNA polymerase (NEB) with the primers CFPA (5'-CACCACGGCCACTGTGGCCATGAGTAAAGGAGAA-GAAGTT-3') and CFPB (5'-GGCCATCGGGGCCCTATTTTATAGTTCATC-CATGCC-3'); topoisomerase-mediated directional cloning requires a 5'-CACC

on the forward primer. Overhanging adenosine residues were added to this product by incubation of 7.5 μ L of the PCR product with 1 μ L of 10X buffer, 1 μ L of 4 mM dNTP, and 0.5 μ L of Taq DNA polymerase at 72°C for 20 min. This reaction was used to clone the *GFPuv* gene into pET100/D-topo, as directed by the manufacturer. The topoisomerase reaction was used to transform chemically competent TOP10 cells, as directed. The resulting plasmid was isolated by miniprep (QIAGEN). pET100GFPuv confers Ap resistance and expresses *GFPuv* under the control of a T7 ribonucleic acid (RNA) polymerase promoter, requiring that a host strain carry a λ DE3 lysogen.

3.2.2. Protein Expression and Purification

To achieve high levels of incorporation of unnatural amino acids, initial growth is carried out in permissive conditions, followed by a switch to conditions that are more restrictive and include inducer (5). This can also be achieved by providing a limited amount of permissive amino acid, and an excess of unnatural amino acid that will be used once the supply of natural amino acid has been exhausted (*see* **Note 3**; **ref. 17**).

3.2.2.1. EXPRESSION AND PURIFICATION OF GST WITH HIGH LEVELS OF INCORPORATION OF 4fW

1. Grow a single colony of C600p transformed with pGSR and selected on Kn plates in M9B1TL95%4fW+Kn overnight.
2. Dilute the starter culture of **step 1** by 1:100 to inoculate a 100-mL culture of the same media. Grow bacteria to mid-log phase (optical density at 600 nm \approx 0.5).
3. Centrifuge the culture at 5400g for 20 min, and resuspend in 100 mL M9B1TL3 \times 100%4fW+Kn supplemented with 0.3 mM IPTG. Grow this culture for a further 16 h (*see* **Note 4**).
4. Centrifuge cells again, as indicated in **step 3** and lyse with 5 mL of B-PER (Novagen) lysing reagent.
5. After centrifugation to remove the insoluble fraction, add 10 mM MgCl₂ (from a 1 M stock solution) and 5 U DNase to the lysate and incubate for 15 min at room temperature.
6. Add 500 μ L of a 50% slurry of glutathione-Sepharose beads to the lysate and mix on a rotator for longer than 2 min at room temperature (**18**).
7. Spin the beads down briefly at maximum speed. Add 5 mL of PBS to wash the beads. Repeat this process twice more.
8. Perform a final wash of 1 mL of ice-cold PBS and transfer to microcentrifuge tubes.
9. Elute purified GST from the beads in three 0.5-mL fractions of 50 mM Tris-HCl, pH 8.0, plus 5 mM reduced glutathione by rotating for approx 2 min at room temperature.
10. Concentrate each fraction by centrifugation using Microcon concentrators.
11. Determine the purity of the proteins by SDS-PAGE; expect greater than 95% purity.

3.2.2.2. EXPRESSION AND PURIFICATION OF *GFP_{UV}* INCORPORATING 6fW

1. Grow C600F(DE3) plus pET100GFP_{UV} in 200 mL of M9B1TLW+Kn+Ap.
2. At mid-log phase, centrifuge cultures and resuspend pellets in 100 mL M9B1TL95%6fW+Kn+Ap with 1 mM IPTG and continue growth for an additional 3 h (*see Note 4*).
3. Spin down cultures and lyse pellets in 3 mL B-PER. Centrifuge the lysate for 30 min at 15,000g to clear insoluble material. To the soluble fraction, add 10 mM MgCl₂ (from a 1 M stock solution) plus 3 U DNase, and allow the lysate to incubate at room temperature for 10 min.
4. Prepare a 3 mL Ni-NTA column (Novagen) by washing with 15 mL water and 9 mL binding buffer.
5. Pass the soluble fraction over the Ni-NTA column. Wash the column with 30 mL binding buffer and 18 mL wash buffer, and elute with 18 mL elution buffer. Collect fractions of 0.5 mL from the column, and analyze by SDS-PAGE and exposure to ultraviolet light (fractions with protein are visibly fluorescent). Pool fractions containing purified protein, and concentrate as described in **Subheading 3.2.2.1**.

3.2.3. Purification of Whole-Cell Protein Extracts

For incorporation of 4fW:

1. Grow the cultures of C600p to saturation in 25 mL of the appropriate media.
2. Pellet the cultures by centrifugation, and lyse in 200 μ L B-PER.
3. Pass 50 μ L of this lysate through a Centri-Sep size-exclusion column to remove unincorporated amino acids.

Similarly, to analyze the level of discrimination of bacteria against 6fW:

1. Grow C600F to saturation in 100 mL of culture on either M9B1TLW+Kn or M9B1TL95%6fW+Kn.
2. Pellet and lyse the bacteria in 200 μ L B-PER II.
3. Pass half of this volume through a Centri-Sep column (*see Subheading 3.3.1.1*).

3.3. Analysis of Incorporation Levels of Unnatural Amino Acids

In this section, two methods are discussed for determining the extent of unnatural amino acid incorporation. Complete hydrolysis followed by HPLC and mass spectrometry (**Subheading 3.3.1.1**) or HPLC followed by HPLC under different conditions (**Subheading 3.3.1.2**) can be used for purified protein and for whole-cell protein extracts. However, protease cleavage and fragment analysis (**Subheading 3.3.2**) can only be used on purified proteins.

3.3.1. Analysis of Purified Proteins

Sample results pertaining to **Subheadings 3.3.1.1** and **3.3.1.2** are presented in **Table 1**.

Table 1
Incorporation of 6fW Into Protein

Method of analysis	Subheading	Incorporation (%)
Whole-cell protein extract		
HPLC-ESI	3.3.1.1	56.5
HPLC-HPLC	3.3.1.2	66.7
Purified GFPuv		
HPLC-ESI	3.3.1.1	68.6
Average incorporation		64.0
From ref. 4.		

3.3.1.1. HYDROLYSIS AND HPLC-ESI ANALYSIS OF PROTEINS CONTAINING UNNATURAL AMINO ACIDS

Acid hydrolysis of protein samples allows for the determination of the makeup of the protein. A global average is achieved, whether for whole-cell protein extracts or for purified proteins.

1. Lyophilize protein samples (e.g., half of the eluant from the Centri-Sep columns in **Subheading 3.2.3.**, or several micrograms of purified protein from **Subheading 3.2.2.**) to dryness.
2. Resuspend pellets in 1 mL of 5.4 M HCl with 10% thioglycolic acid to preserve tryptophan during hydrolysis.
3. Perform hydrolysis overnight, under a vacuum, at 110°C.
4. Lyophilize the hydrolysates again, and resuspend in 50 μ L of water.

Hydrolysates can be analyzed by HPLC-ESI. In this case, the specific mass of the natural and unnatural amino acid can be detected and followed as they elute from the HPLC column. The relative ratios of the eluted masses can be determined as areas under the curves, and represent the relative amount of natural and unnatural amino acids. The actual molar amounts of amino acids can also be determined by generating a standard curve.

3.3.1.2. HPLC-HPLC ANALYSIS OF HYDROLYZED PROTEIN SAMPLES

An alternative approach is to run serial HPLC samples.

1. Inject hydrolysates onto a C-18 column and elute with the following program:
 - a. 94% Buffer A and 6% Buffer B for 20 min.
 - b. Switch by gradient to 98% Buffer A and 2% Buffer B during 10 min.
 - c. Elute for an additional 15 min.
 - d. Re-equilibrate the column to 94% Buffer A and 6% Buffer B in the last 5 min of the program.

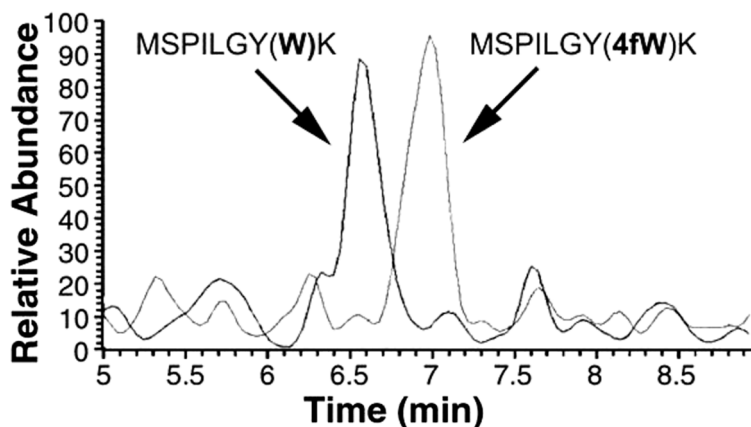


Fig. 2. Relative incorporation of 4fW and W. GST purified from C600pGSR grown in 95% 4fW was digested with trypsin and analyzed by HPLC-ESI. The peptide MSPILGY(W/4fW)K produces two peaks at masses 1094 and 1112 daltons, of roughly equivalent proportion (Data is reproduced with permission from **ref. 5**.)

2. Peaks eluting at times corresponding to standards (e.g., W and 6fW) are collected and lyophilized, and then resuspended in water.
3. Reinject samples on the same column, but under the buffer system of 80% buffer C and 20% Buffer D. Compare the elution times to standards.

The use of the reinjection strategy allows a confirmation of identity; coelution of different molecules may occur under one set of buffer conditions, but is unlikely under two. The area under the curve of the second buffer condition may be considered as a pure sample, and comparisons of these areas may be made between peaks corresponding to natural and unnatural amino acids.

In the case of tryptophan and tryptophan analogs, detection is made at 280 nm, without the need for derivatization (*see Note 5*). Phenylalanine and tyrosine give minor peaks from purified protein, whereas a large number of minor peaks are present in hydrolysates from whole-cell protein extracts. Nevertheless, quantitative data can be obtained.

3.3.2. Protease Digestion to Determine Unnatural Amino Acid Incorporation Levels

Because the masses of specific peptide fragments can be predicted based on the sequence of the protein, mass shifts in the predicted fragments are likely caused by the incorporation of the specific unnatural amino acid in question. As such, analysis of protease digestion products is a sensitive method for the determination of amino acid analog incorporation (**5,19,20**).

1. Purified GST (for example, 5 μg , e.g., from **Subheading 3.2.2.1.**) is lyophilized and resuspended in 0.1 M NH_4HCO_3 .
2. Digest with immobilized L-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Pierce) at 37°C for 10 h.
3. Remove trypsin by centrifugation (*see Note 6*).
4. Lyophilize the digest, and resuspended in water to 210 μM .
5. Analyze digestion products by HPLC-ESI.

Elution profiles of specified masses can be followed, and demonstrate the level of incorporation of unnatural amino acids into specific fragments (**Fig. 2**).

4. Notes

1. Unnatural amino acids are not often commercially available in an enantiomerically pure form. In the case of a racemic mixture, 20 $\mu\text{g/mL}$ of the L-enantiomer should be used. If an unnatural amino acid is being mixed with a natural amino acid, double the amount of unnatural amino acid to be used, if it is racemic. For example, 95% 4fW requires 38 $\mu\text{g/mL}$ DL-4fW plus 1 $\mu\text{g/mL}$ L-W (**5**).
2. Auxotrophy of the bacterial strain to be used for incorporation is preferable, but may not be necessary for all applications. To select a strain that exhibited genetic code ambiguity, Döring et al. used a strain that was prototrophic for valine and cysteine, and selected variants that could substitute cysteine for valine at a valine codon; the result was an editing-deficient valyl-transfer RNA synthetase (**19**).
3. The vectors discussed here are typical of expression plasmids. One uses the T7 RNA polymerase system and is under the control of the lac operator, whereas the other directly uses the lac promoter for expression of targeted genes. Many other expression vectors have been used for the incorporation of unnatural amino acids into proteins of interest. Furthermore, these have been conducted for full, partial, and site-specific incorporation of the analog (**1,5,19**).
4. Optimization may be required to achieve maximal expression and incorporation of a gene of interest. Critical factors for optimization include the timing between inoculation of the culture and the switch to media with the inducer and unnatural amino acid, and the length of time of expression. For example, it was found that the expression of GST was maximal from a mid-log culture with overnight expression, whereas GFPuv was best expressed from a mid-log culture for only several more hours. More strikingly, the expression of enhanced cyan fluorescent protein for fluorescent assays, including fluorescence-activated cell sorting, was best achieved by inducing an overnight culture to express protein for 6 h (J. M. Bacher and A. D. Ellington, unpublished observations).
5. Analysis of hydrolyzed proteins by HPLC is simplified in these examples by examining the products for tryptophan, a naturally absorbing amino acid. If performing similar experiments for other amino acid analogs, the amino acids may require derivatization before HPLC analysis.
6. Trypsin treatment can effectively be achieved by performing the reaction in a Millipore spin column with a 0.45- μm pore-size filter (M. P. Robertson, personal communication, May 12, 2003). At the end of the reaction time, the reaction can be

purified of trypsin by centrifugation. The immobilized trypsin is trapped on the membrane, whereas the eluate carries the reaction products.

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