Chapter 2

NON-CANONICAL AMINO ACID SUBSTRATES OF THE L,F-TRANSFERASE

Portions of the work presented here are taken from:

Connor, R.E., Piatkov, K., Varshavsky, A., and D.A. Tirrell, "Enzymatic N-terminal addition of noncanonical amino acids to peptides and proteins." *ChemBioChem*, 2008, in press.

Abstract: A one-pot, fully enzymatic method for the in vitro N-terminal labeling of peptides with non-canonical amino acids has been developed using the L,F transferase (Aat) from *E. coli*. An assay using a model substrate and subsequent HPLC analysis was used to identify candidate aminoacyl-tRNA substrates for the transferase. We have shown that Aat is highly permissive in its requirements for aminoacyl-tRNA substrates and that the enzyme can catalyze the transfer of leucine, methionine, tryptophan, and phenylalanine analogs. The aminoacyl-tRNA substrates are produced in situ by aminoacyl-tRNA synthetases that will aminoacylate a tRNA with the desired amino acid. This system utilizes previously designed mutant and wild-type synthetases that efficiently charge non-canonical amino acids onto native tRNA. Although the L,F-transferase is a bacterial enzyme, it will utilize aminoacyl-tRNA from both *E. coli* and *S. cerevisiae*. The leucine analog, azaleucine, and phenylalanine analogs, 3,2- and 3,3- pyridylalanine, were not successfully appended by the L,F-transferase despite the known activity of the wild-type leucine and phenylalanine tRNA-synthetases towards these analogs.

2.1 Introduction

The incorporation of radiolabeled leucine into the protein fraction of ribosome-free cell extracts of bacteria was first reported in 1963 by Kaji, Kaji and Novelli.[1] This surprising result led to the identification of the enzyme known as the leucyl, phenylalanyl-tRNA transferase (L,F-transferase), which effects the post-translational modification of proteins with amino acids.[2] After identification and purification, the enzyme was characterized biochemically and found to specifically modify lysine and arginine residues on the N-termini of known purified proteins with leucine or phenylalanine.[3, 4] The L,F-transferase catalyzes the exchange of an amino acid from an aminoacyl-tRNA to a substrate protein bearing a basic N-terminus. The transferase requires the synthesis of the aminoacyl-tRNA by an aminoacyl-tRNA synthetase, the same enzymes that charge tRNAs for use in protein synthesis.

Before the gene for the transferase was identified, *E. coli* knockout strains were produced to attempt to elucidate the role of the L,F-transferase in bacterial metabolism.[5] The transfer of methionine *in vitro* by the L,F-transferase was also noted.[6] The gene encoding the L,F-transferase, *aat*, was identified and cloned in 1993 by Alex Varshavsky's laboratory.[7] It was subsequently over-expressed in *E. coli* and affinity-purified by Thomas Shrader and co-workers.[8] Using over-expressed transferase, the enzyme's inability to use valine as an aminoacyl substrate on both $tRNA^{Val}$ and $tRNA^{Met}$ was confirmed.[3, 9] However, the misacylation of $tRNA^{Val}$ with methionine did result in methionyl transfer to a protein substrate, which further supported the hypothesis that the L,F-transferase requires an unbranched beta carbon in its aminoacyl-tRNA substrates. The permissivity of the L,F-transferase towards the tRNA is demonstrated by its use of not only

$tRNA^{Leu}$ and $tRNA^{Phe}$, but also $tRNA^{Met}$, $tRNA^{Val}$. Moreover, disruption of the secondary structure of aminoacyl-tRNAs through invasion with deoxyoligonucleotides has shown that tRNA secondary structure is not required for amino acid transfer. Only an unpaired 3' overhang and a portion of the acceptor stem of the tRNA are required for recognition by the L,F-transferase.[9]

The function of the L,F-transferase in cellular metabolism was unknown until it was discovered to be part of the N-end rule in bacteria by Varshavsky and coworkers.[10] N-end rule pathways in both prokaryotes and eukaryotes lead to protein degradation via ATP dependent proteolysis based upon the N-terminal residue of the protein.[10, 11] The L,F-transferase, ATP-dependent protease ClpAP, and the adapter protein, ClpS, comprise the *E. coli* N-end rule pathway.[12, 13] In *E. coli*, the primary destabilizing residues are leucine, phenylalanine, tryptophan, and tyrosine, which are directly recognized by chaperones ClpS and ClpA before being shuttled to ClpP for degradation.[13, 14] The L,F-transferase transforms proteins bearing "secondary" destabilizing N-terminal residues arginine and lysine into N-degrons by appending N-terminal leucine or phenylalanine.[10]

Recently, the *E. coli* L,F-transferase has been used to modify proteins with phenylalanine analogs delivered from chemically synthesized aminoacyl-tRNAs.[15, 16] Here, a fully enzymatic method for the N-terminal introduction of non-canonical amino acids to proteins and peptides is described. Enzymatically aminoacylated tRNAs can be utilized by the L,F-transferase to deliver leucine, phenylalanine, methionine, and tryptophan analogs to substrate peptides. A simple chromatographic assay was used to demonstrate transferase-mediated introduction of the non-canonical amino acids shown in Scheme 2-1 to the acceptor peptide lysylalanyl-7-amino-4-methylcoumarin (KA-AMC).

The analysis of the product peaks by electrospray ionization mass spectrometry was used to verify the identity of each tripeptide product as shown in Figure 2-1. The mass spectrometry data is given in Appendix I of this thesis. Using the HPLC-based assay, the ability of the L,F-transferase to utilize aminoacyl-tRNA from yeast was explored.

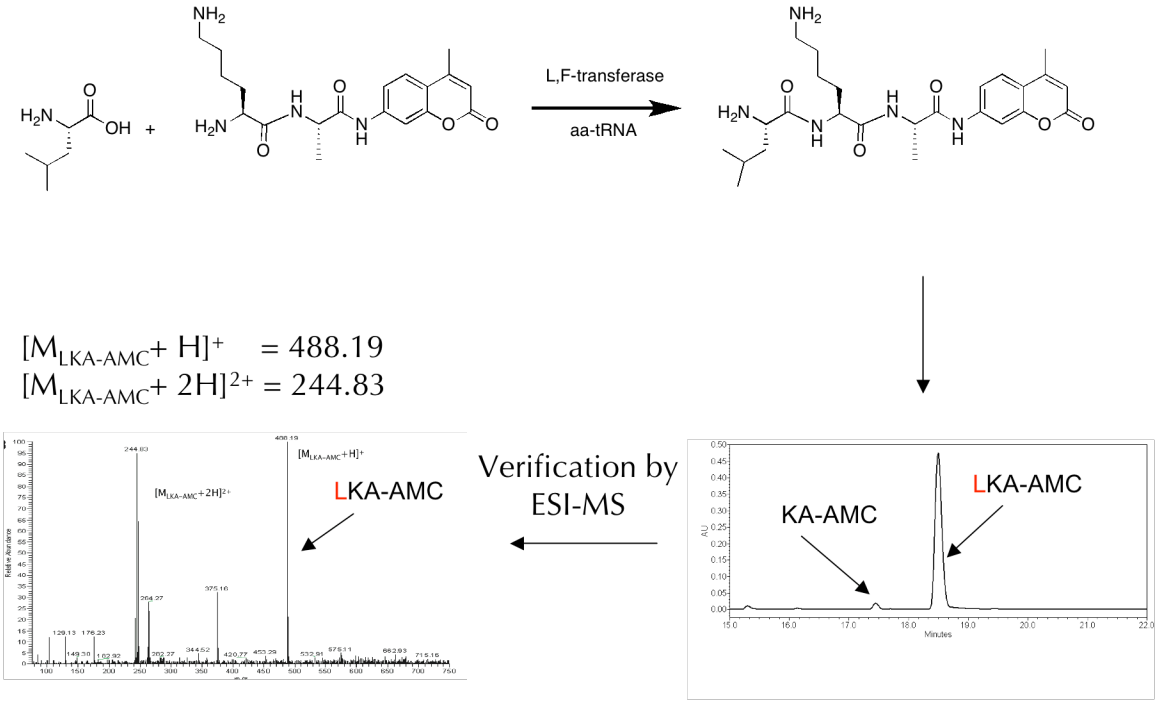
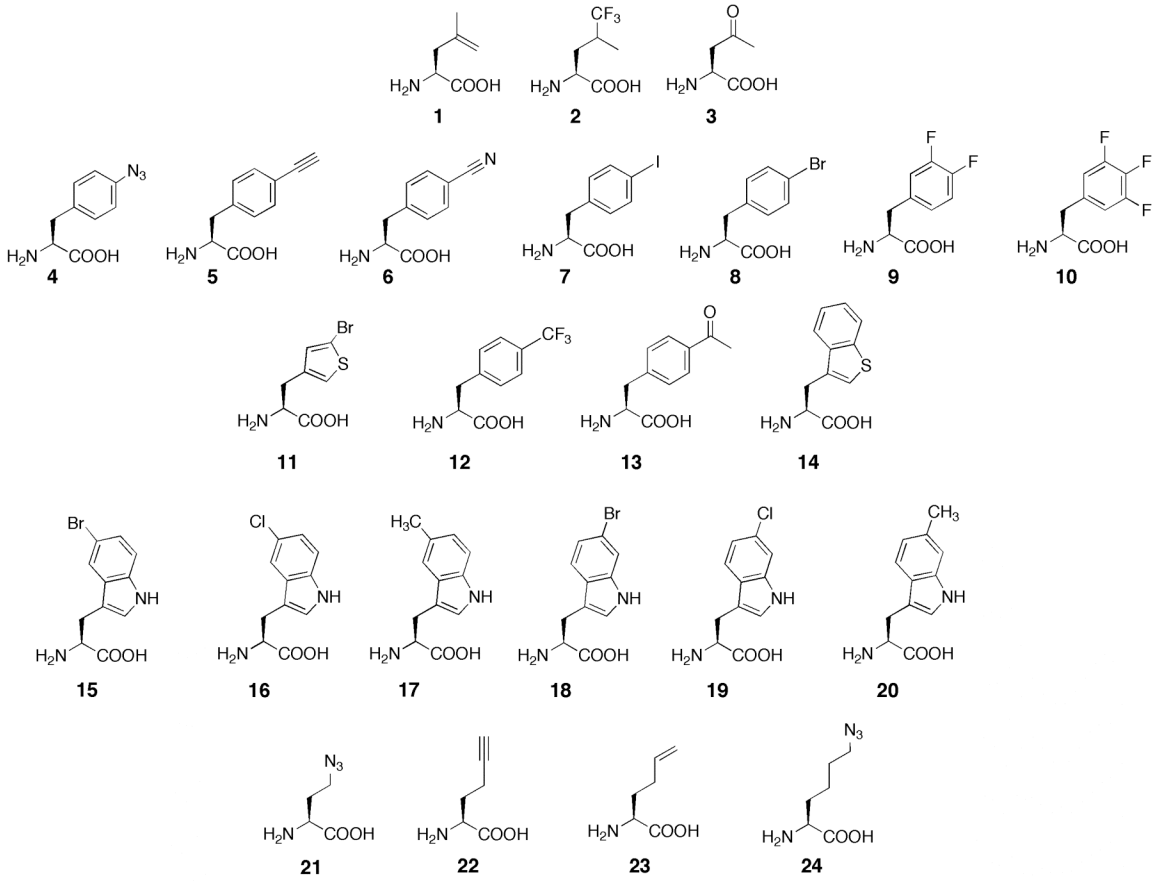


Figure 2-1. Illustration of the assay for transfer of aminoacyl-tRNA substrates to a dipeptide-coumarin by the L,F-transferase.



Scheme 2-1. Non-canonical amino acids tested as substrates for the L,F-transferase.

2.2 Materials and Methods

2.2.1 Materials

Chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification, unless otherwise noted. The substrate dipeptide, Lys-Ala-4-aminomethylcoumarin, and amino acids **4** and **6** were purchased from Bachem (Bubendorf, Switzerland) and used as received. Amino acids **12**, **14**, 3,3-pyridylalanine, and 3,2-pyridylalanine were purchased from Chem Impex (Wood Dale, IL). Azaleucine, **7**, **8**, **15**, **17**, and **20** were also purchased from Sigma-Aldrich. Amino acids **9**, **10**, and **11** were obtained from Peptech Corp. (Burlington, MA), and **16**, **18**, and **19** were purchased from Biosynth (Staad, Swirzerland). Amino acid **2** was purchased from Fluorochem (Derbyshire, UK); **1**, **3**, **22**, and **23** were prepared by alkylation of diethyl acetamidomalonate with the appropriate tosylate as described previously.[17] Compounds **21** and **24** were synthesized by diazo transfer to the equivalent amine-bearing amino acids;[18] **5** and **13** were prepared as previously reported.[19, 20] The *E. coli* phenylalanyl, leucyl- and methionyl-tRNA synthetases were produced as described.[19, 21] The leucyl, phenylalanyl transferase was purified as previously described.[8]

2.2.2 Reaction conditions for chromatographic analysis

Each 50 mL reaction mixture contained amino acid (1 mM), cognate synthetase (400 nM), total *E. coli* tRNA or total *S. cerevisiae* tRNA (30 mg, 11.5 pmol), Aat (800 nM) and KA-AMC (100 μM) in the transferase reaction buffer (50 mM Tris, pH 8.0; 50 mM β-mercaptoethanol; 5 mM ATP; 10 mM $MgCl_2$; 150 mM KCl). The reaction mixtures were

incubated at 37°C and stopped either by filtration through a Microcon YM-10 filter or by precipitation of the proteins with acetone. After centrifugation through the YM-10 filter and 2-3 washes (100 μl) of water, the filtrate was separated by HPLC, and used for subsequent MALDI analysis. If precipitation was used to remove the added synthetase and transferase, 4 volumes of acetone were added and the reaction mixture incubated at -20°C for at least 1 h. The resulting precipitate was sedimented by centrifugation and the supernatant, containing both the substrate and product, was transferred to a new tube, concentrated and analyzed by HPLC.

2.2.3 HPLC separation of tripeptide products from KA-AMC

All liquid chromatography, except that on *p*-azidophenylalanine and tryptophan reaction mixtures, was performed on a Waters HPLC system with a Microsorb C18 column (Varian, Inc). The buffers used for separation of the tripeptide products were 0.1% trifluoroacetic acid (Eluent A) and 100% acetonitrile (Eluent B). The gradient consisted of 0-5 min, 0% B; 5-10 min, 0-30% B; 10-25 min, 30-60% B, 25-30 min, 60-100% B, 30-40 min, 100% B. Dual detection at 214 and 324 nm was used to identify the substrate; product peaks and fractions containing the product were collected for analysis by electrospray ionization mass spectrometry. For oxonorvaline, a modified gradient was required to separate the substrate from the product tripeptide. The gradient was 0-5 min, 0% B; 5-10 min, 0-24% B; 10-25 min, 24-48% B; 25-40 min, 48-100% B; 40-45 min, 100% B. The *p*-azidophenylalanine reaction mixture was separated on a Varian HPLC system with a Microsorb C18 column. The buffers for separation were 0.1% trifluoroacetic acid and 80% acetonitrile, 0.06% trifluoroacetic acid. The gradient consisted of 0-5 min, 0% B; 5-7 min, 0-30% B; 7-27

min, 30-100% B; 27-37 min, 100% B. Detection at 324 nm was used to identify the substrate and product peaks.

2.3 Results and Discussion

Each amino acid shown in Scheme 2-1 was tested in a one-pot reaction containing an aminoacyl-tRNA synthetase and L,F-transferase. In this method, the required aminoacyl-tRNA substrates were prepared *in situ* by treatment of the amino acids of interest with their cognate *E. coli* aminoacyl-tRNA synthetases. *In situ* production of the aminoacyl-tRNA requires no intermediate purification steps and furthermore, some purified wild-type *E. coli* synthetases are commercially available. After each test reaction, the proteins were precipitated with acetone and the supernatant containing both the peptide substrate and product was retained and dried. The dried peptides were then re-suspended in water, separated on a reverse phase C18 HPLC column, and then fractionated for verification by ESI mass spectrometry.

2.3.1 Leucine Analogs

The leucine analogs, 4-dehydroleucine (**1**) and 5,5,5-trifluoroleucine (**2**) were both charged to tRNA by the wild-type *E. coli* leucyl-tRNA synthetase (LeuRS) for use by the L,F-transferase.[21, 22] Figure 2-2 contains HPLC traces for amino acids **1**, **2**, and **3** after four hours of incubation in the transferase reaction. Amino acid **2** is a particularly good analog for leucine and is transferred completely in four hours. In the trace, the two isomers of **2** are slightly separated resulting in a bifurcated product peak. An editing mutant of

LeuRS, T252Y, allows the misacylation of $tRNA^{Leu}$ with **3** as well as methionine analogs.[21] This ketone analog of leucine, oxonorvaline (**3**), offers an opportunity for biochemical modification through hydrazide-ketone coupling and transferred after a four hour reaction.

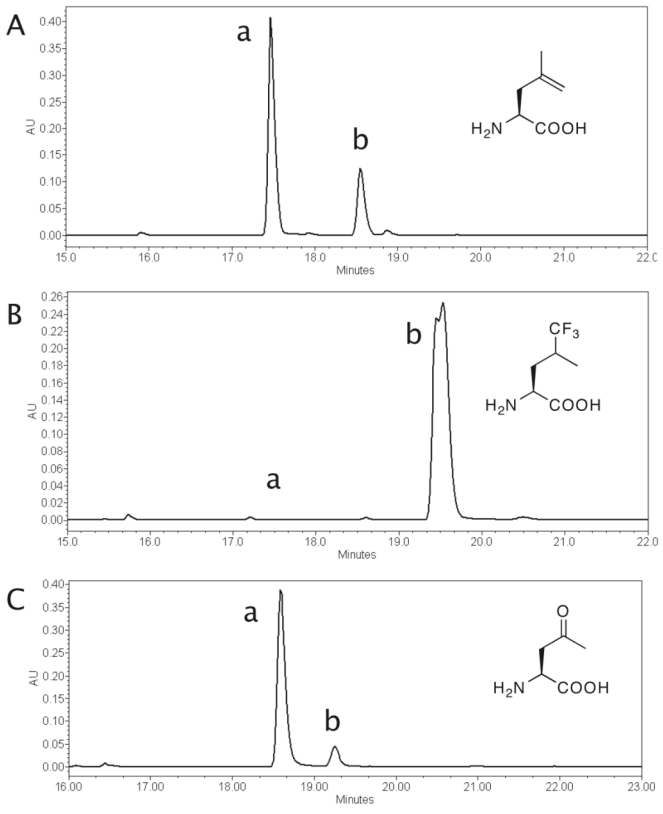


Figure 2-2. HPLC analysis of the transfer of leucine analogs **1**, **2**, and **3** to the dipeptide substrate. A) HPLC analysis of the transfer of **1** to KA-AMC after charging by wild-type LeuRS. B) Chromatograph indicating the complete transfer of **2** to KA-AMC after charging by the wild-type LeuRS. C) HPLC analysis of the transfer of **3** to KA-AMC after charging by the T252Y mutant LeuRS. For all traces, peak **a** is KA-AMC and peak **b** is the tripeptide product. Mass spectra are available for compounds **1b**, **2b**, and **3b** in Appendix I.

2.3.2 Phenylalanine Analogs

Amino acids *p*-azidophenylalanine (**4**), *p*-ethynylphenylalanine (**5**), and *p*-cyanophenylalanine (**6**) were charged to *E. coli* tRNA using the A294G mutant of the *E. coli* phenylalanyl-tRNA synthetase (PheRS). This mutation was originally identified by Ibba and Henneke for the incorporation of *p*-chlorophenylalanine into *E. coli* proteins.[23] The utility of the mutant synthetase was further expanded by Kirshenbaum et al. with the discovery that it will aminoacylate *E. coli* $tRNA^{Phe}$ with a variety of non-canonical amino acids.[19] The two reactive analogs, **4** and **5,** are transferred in almost quantitative yields after four hour reaction incubation. The aryl azide analog, **4**, can be used as a photocrosslinking agent as well as a partner in azide-alkyne ligations.[24-26] *p*-Cyanophenylalanine is also transferred, as shown in Figure 2-3.

A variety of halogenated phenylalanine analogs are also charged by the A294G PheRS and used by the L,F-transferase for modification of the dipeptide substrate. Figure 2-4 contains HPLC traces after four hour reactions of *p*-iodophenylalanine (**7**), *p*-bromophenylalanine (**8**), 3,4-difluorophenylalanine (**9**), and 3,4,5-trifluorophenylalanine (**10**). Amino acids **7**, **8**, and **9** are transferred in near quantitative yield. Fluorinated analogs are capable of changing the chemical nature of proteins, and they have been shown to enhance the dimerization and thermostability of leucine zippers.[22, 27, 28] The chemical addition of highly fluorinated tags onto proteins has been used as a strategy for purification using fluorous phase chromatography.[29] The halogenated amino acids, **7** and **8** can be used for palladium-catalyzed reactions, such as Sonogashira or Heck couplings.[30]

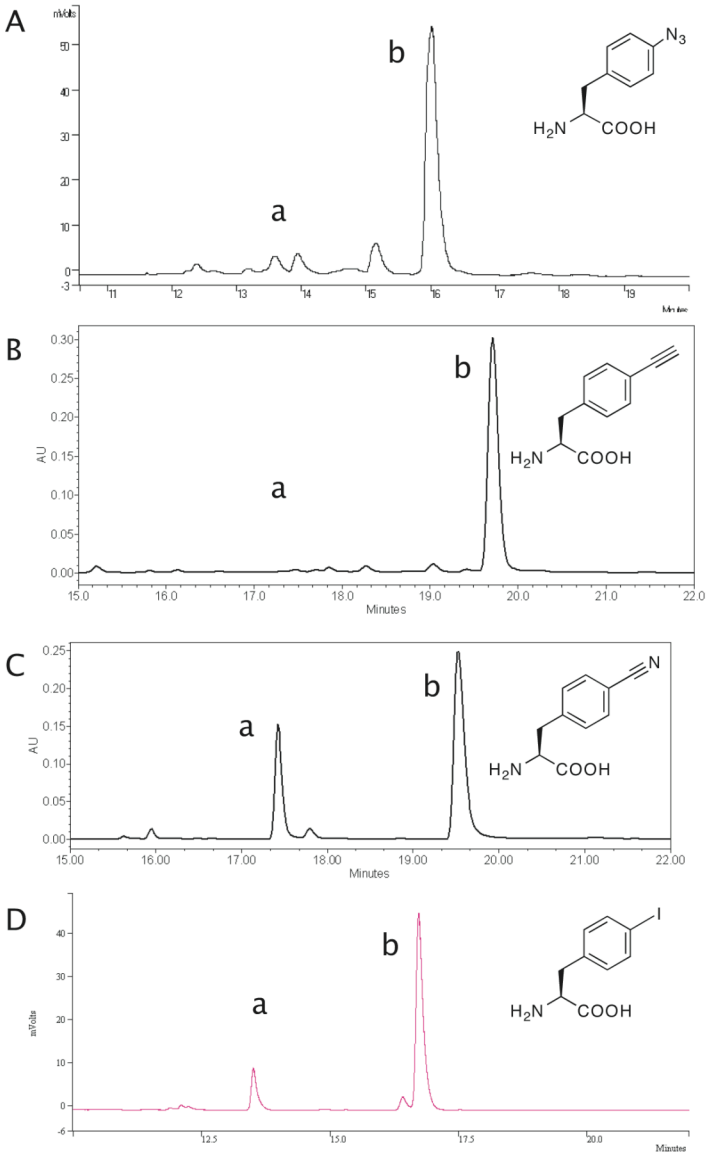


Figure 2-3. HPLC analysis of the transfer of phenylalanine analogs **4**, **5**, **6**, and **7** to the dipeptide substrate. A) Chromatgraph indicating the complete transfer of **4 to** KA-AMC after charging by the A294G PheRS. B) Analog **5** is also completely transferred to KA-AMC after charging by the A294G PheRS. C) HPLC analysis of the transfer of **6** to KA-AMC after charging by the A294G PheRS. D) Almost complete conversion to the product tripeptide is shown with charging of **7** by the A294G PheRS. For all the traces, peak **a** is KA-AMC and peak **b** is the tripeptide product. Mass spectra are available for compounds **4b**, **5b**, **6b**, and **7b** in Appendix I.

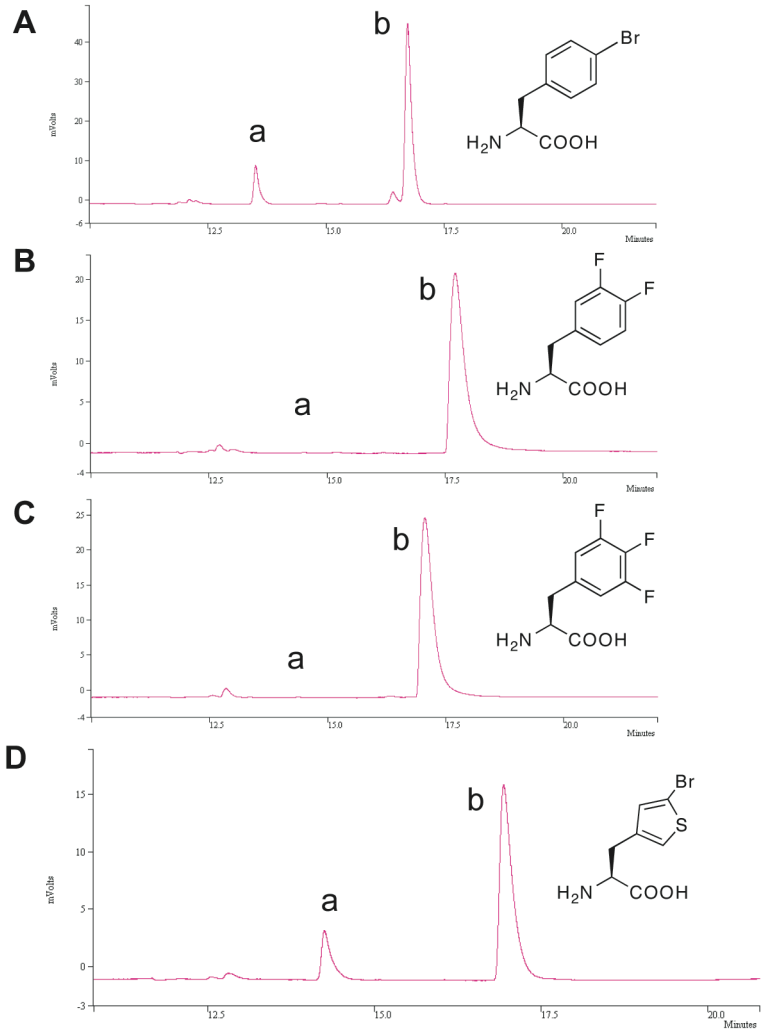


Figure 2-4. HPLC analysis of the transfer of phenylalanine analogs **8**, **9**, **10**, and **11** to the dipeptide substrate. A) Transfer of **8** to KA-AMC with charging by the A294G PheRS is almost complete after four hours. B) HPLC analysis indicating that complete conversion to tripeptide product occurs after four hours with **9** being charged by the A294G PheRS. C) Complete conversion to tripeptide product is also shown for **10** charged by the A294G PheRS. D) Brominated thiophene analog **11** is transferred to KA-AMC after charging by the A294G PheRS. Peak **a** is KA-AMC and peak **b** is the tripeptide product. Mass spectra are available for compounds **8b**, **9b**, **10b**, and **11b** in Appendix I.

2.3.3 Aminoacylation and transfer of large phenylalanine and tryptophan analogs

A computationally designed A294G/T251G PheRS mutant originally used to generate *p*-acetylphenylalanyl-tRNA allows a broad range of large phenylalanine and tryptophan analogs to be charged onto *E. coli* $tRNA^{Phe}$.[21] Figure 2-5 shows the transfer of analogs *p*-trifluoromethylphenylalanine (**12**), *p*-acetylphenylalanine (**13**), and benzothienylalanine (**14**) by the L,F-transferase using the PheRS A294G/T251G mutant synthetase to generate the aminoacyl-tRNA substrates. Halogenated and methylated tryptophan derivatives can also be charged to $tRNA^{Phe}$ using the A294G/T251G PheRS. The halogenated tryptophan analogs offer not only a scaffold for palladium coupling reactions, but also potential to modify the spectral properties of the protein substrate.[30, 31] HPLC analyses of the transfer of tryptophan derivatives bearing substituents at position 5 on the indole ring (5-bromotryptophan (**15**), 5-chlorotryptophan (**16**), and 5-methyltryptophan (**17**)) are shown in Figure 2-6. The three 6-substituted analogs, 6-bromotryptophan (**18**), and 6-chlorotryptophan (**19**), and 6-methyltryptophan (**20**) were also successfully transferred by Aat, as illustrated in Figure 2-7. Using site-specific *amber* suppression with a yeast tRNA/ synthetase pair, **18** and **19** were individually added to the chromophore of GFP to alter its fluorescence; however, **15** was found to be much less translationally active than implied by its kinetic values from activation assays.[31]

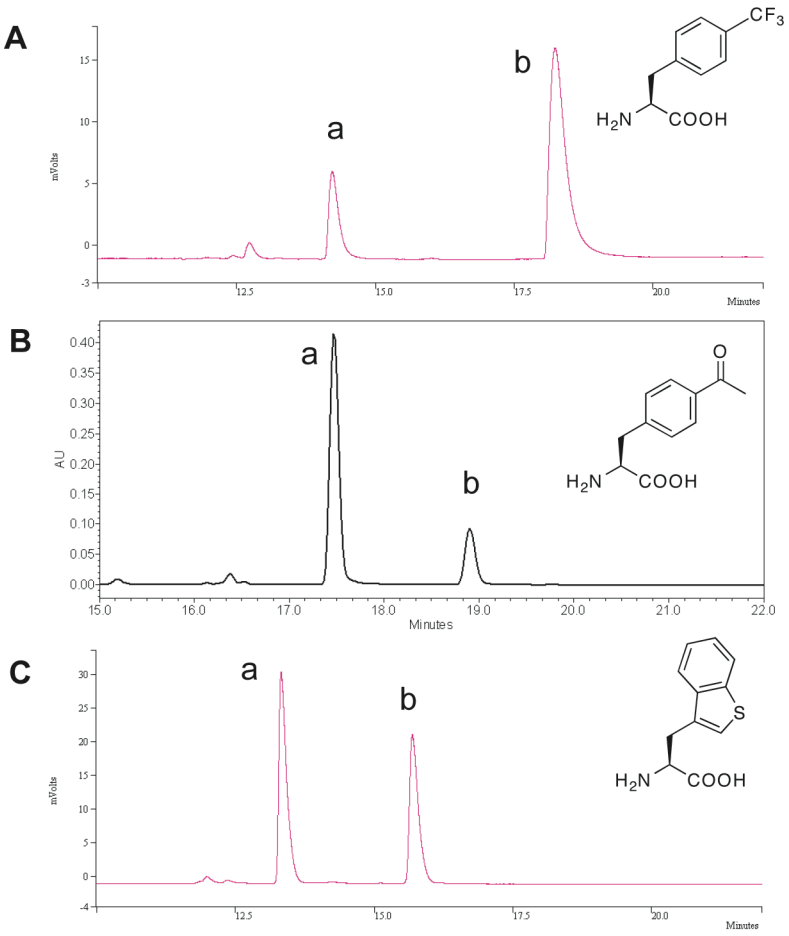


Figure 2-5. HPLC analysis of the transfer of analogs **12**, **13**, and **14** to the dipeptide substrate. A) HPLC analysis of the transfer of **12** to KA-AMC after four hours with charging by the A294G/T251G PheRS. B) HPLC analysis of the transfer of **13** to KA-AMC after four hours with charging by the A294G/T251G PheRS. C) Tryptophan analog **14** is charged by the A294G/T251G PheRS and transferred to KA-AMC after four hours. In all traces, peak **a** is KA-AMC and peak **b** is the tripeptide product. Mass spectra are available for compounds **12b**, **13b**, and **14b** in Appendix I.

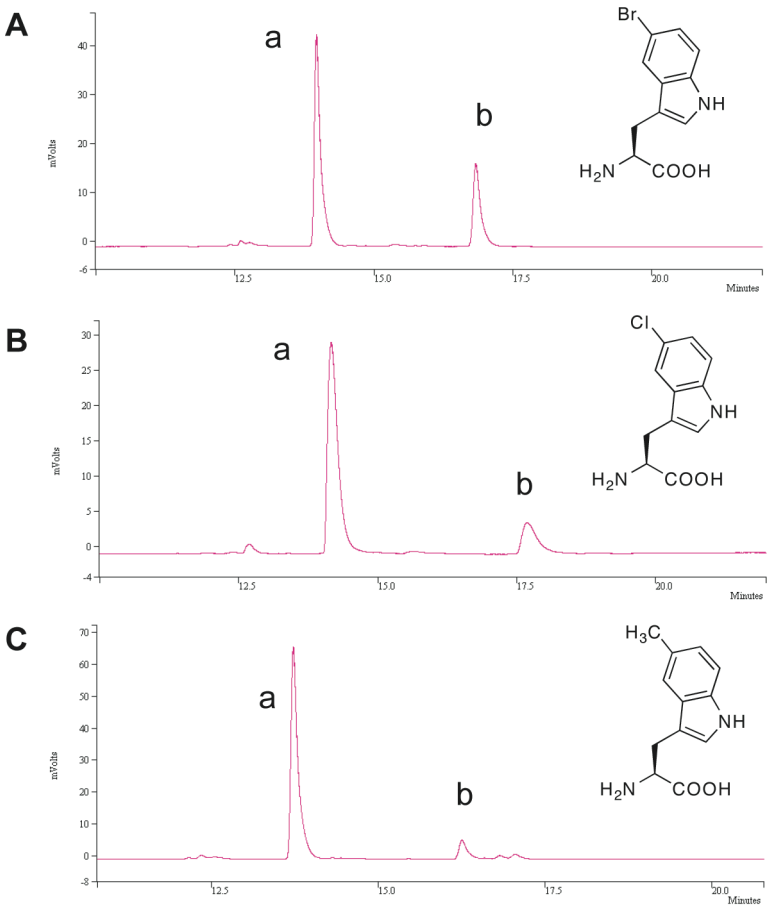


Figure 2-6. HPLC analysis of the transfer of phenylalanine analogs **15**, **16**, and **17** to the dipeptide substrate. A) Chromatograph illustrating the transfer of **15** to KA-AMC after charging by the A294G/T251G mutant PheRS. B) HPLC analysis of the transfer of **16** to K-AMC after charging by the A294G/T251G PheRS. C) Analysis of the tripeptide product resulting from the charging of **17** with the A294G/T251G PheRS. In all traces, peak **a** is KA-AMC and peak **b** is the tripeptide product. Mass spectra are available for compounds **15b**, **16b**, and **17b** in Appendix I.

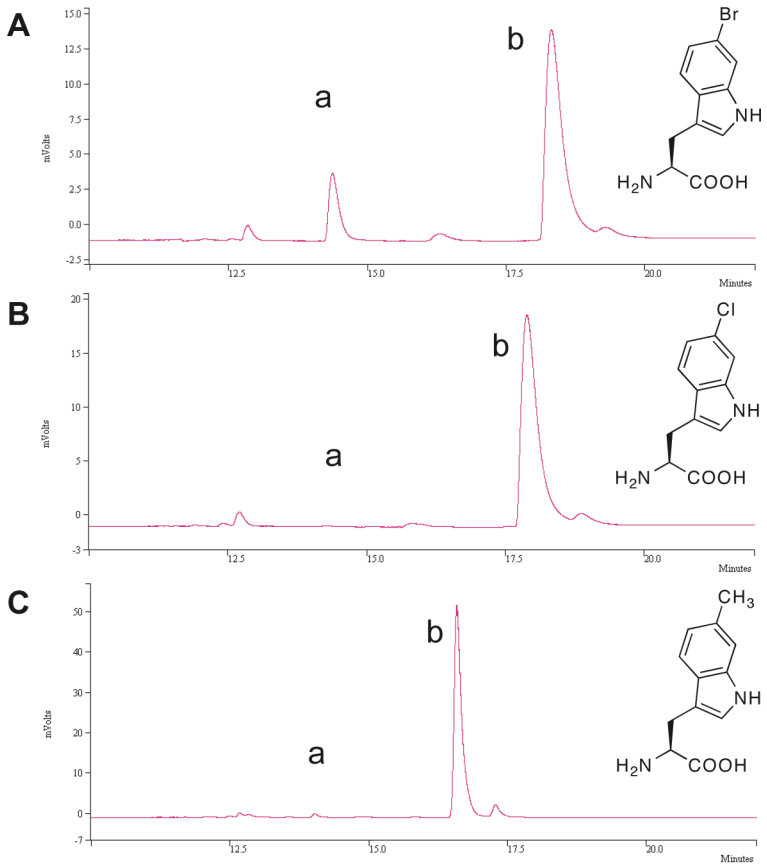


Figure 2-7. HPLC analysis of the transfer of 6-substituted tryptophan analogs **18**, **19**, and **20** to the dipeptide substrate. A) Chromatograph showing the transfer of **18** to KA-AMC after charging by the A294G/T251G PheRS. B) Analog **19** is completely transferred to KA-AMC after 4 hours with charging by the A294G/T251G PheRS. C) Chromatograph indicating the quantitative modification of KA-AMC with **20** after charging by the A294G/T251G PheRS. In all of the traces, peak **a** is KA-AMC and peak **b** is the tripeptide product. Mass spectra are available for compounds **18b**, **19b**, and **20b** in Appendix I.

2.3.4 Methionine Analogs

A variety of methionine surrogates have been incorporated into proteins in *E. coli* using wild-type methionyl tRNA-synthetases (MetRS).[17, 32, 33] Three methionine analogs containing bioorthogonal moieties of interest, azidohomoalanine (**21**), homopropargylglycine (**22**), and homoallylglycine (**23**), can be aminoacylated by wild-type MetRS and subsequently transferred by the L,F-transferase (Figure 2-8). These analogs offer azide, alkene, and alkyne reactive groups that can be accessed with chemistries such as azide-alkyne ligation, Staudinger ligation, and palladium-catalyzed couplings.[25, 30, 34-37] A larger azide-bearing methionine analog, azidonorleucine (**24**), is not translationally active in wild-type *E. coli*; however, a mutant MetRS has been discovered that will allow the ribosomal incorporation of **24** into proteins.[38] The L13G mutant MetRS was used to aminoacylate $tRNA^{Met}$ with **24** for transfer of the amino acid by the L,F-transferase, as shown in Figure 2-7.

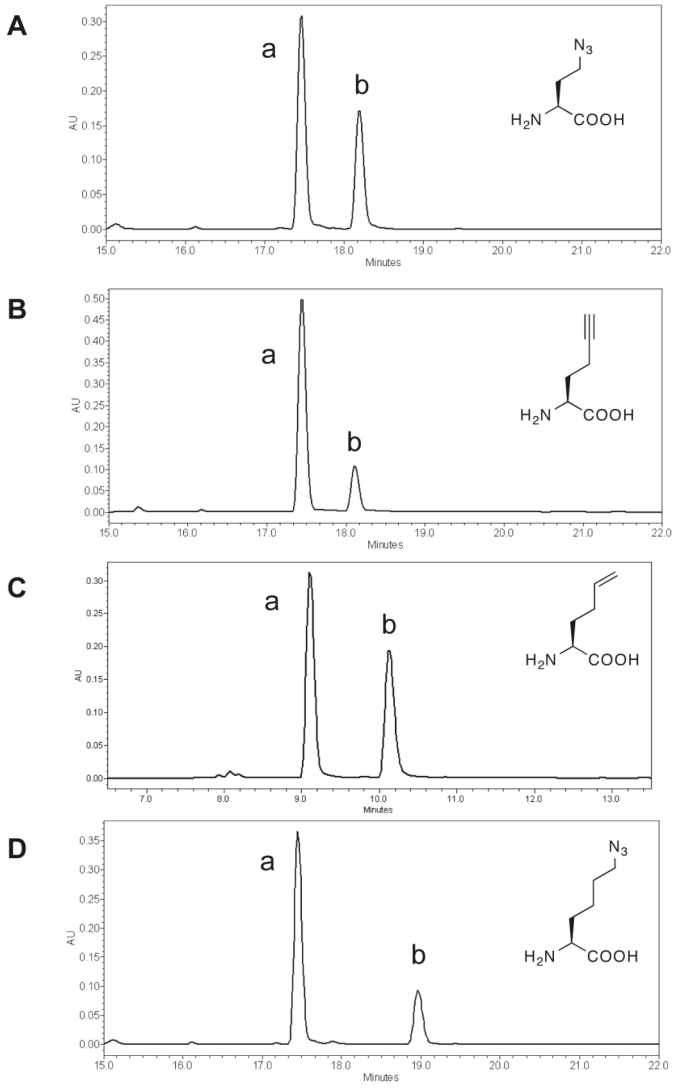


Figure 2-8. HPLC analysis of the transfer of methionine analogs **21**, **22**, **23**, and **24** to the dipeptide substrate. A) Analysis of the transfer of **21** to KA-AMC after charging by the wild-type MetRS. B) HPLC analysis of the transfer of **22** to KA-AMC after charging by the wild-type MetRS. C) HPLC analysis of the transfer of **23** to KA-AMC after charging by the wild-type MetRS. D) Analysis of the transfer of **24** to KA-AMC after charging by the L13G MetRS. In all the trances, peak **a** is KA-AMC and peak **b** is the tripeptide product. Mass spectra are available for compounds **21b**, **22b**, **23b,** and **24b** in Appendix I.

2.3.5 Amino acid transfer from S. cerevisiae *tRNA*

The requirements of the L,F-transferase for its aminoacyl-tRNA substrates are minimal. The enzyme has been shown to accept *in vitro* transcribed tRNAs, tRNAs stripped of secondary structure, and even a single nucleoside attached to phenylalanine.[9, 39] The transferase does show some preference for different tRNAs; for example, the rate of transfer from initiator $tRNA^{Met}$ is slower than the rate from elongator $tRNA^{Met}$.[6] In order for the L,F-transferase to access the aminoacylated acceptor stem of a folded tRNA, the paired portion of the helical stem must be unwound, implying that the difference in transfer rate among tRNAs may arise from differences in G-C base pair content in the acceptor stem.[9] Due to the predominance of the aminoacyl moiety in the recognition elements for aminoacyl-tRNA substrates of the L,F-transferase, we predicted that the L,F-transferase might be able to use the *S. cerevisiae* $tRNA^{Phe}$ to append natural and non-canonical amino acids to peptides. A yeast PheRS had previously been developed for the aminoacylation of a yeast suppressor tRNA with 2-napthylalanine for site-specific introduction into proteins in *E. coli*.[40] This PheRS, a single T415G mutant, can also aminoacylate wild-type yeast $tRNA^{Phe}$ with a variety of other phenylalanine and tryptophan analogs.[31, 41]

As shown in Figure 2-9, the L,F-transferase will use aminoacylated yeast $tRNA^{Phe}$ as a substrate for modification of a model dipeptide. The extent of transfer from a yeast tRNA using the T415G mutant of the yeast PheRS for analogs **11**, **12**, and **14** is demonstrated by the HPLC traces in Figure 2-9. The yeast mutant synthetase has

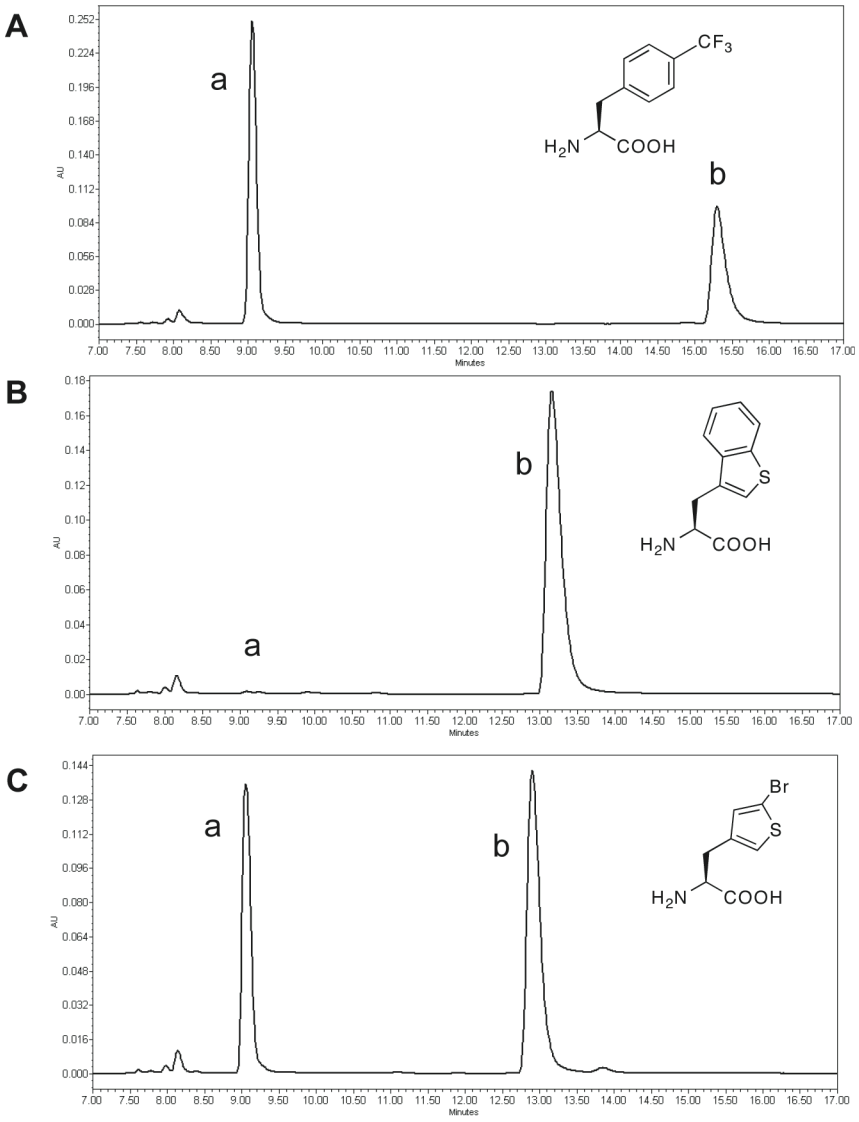


Figure 2-9. HPLC analysis of the transfer of tryptophan analogs **12**, **14**, and **11** to KA-AMC. A) Analysis of the transfer of **12** to KA-AMC after charging by the T415G yeast PheRS. B) HPLC Analysis of the complete transfer of **14** to KA-AMC after charging by the T415G yeast PheRS. C) Analysis of the transfer of **11** to KA-AMC after charging by the T415G yeast mutant PheRS. In all traces, peak **a** is KA-AMC and peak **b** is the tripeptide product.

higher affinity for the 5-substituted tryptophan analogs than the *E. coli* double mutant PheRS, as indicated by the yield of the reactions analyzed in Figure 2-10. The 6-substituted tryptophan analogs are also charged by the T415G yeast PheRS and transferred by the L,F-transferase (Figure 2-11).

2.3.6 Incompatible analogs

Although the L,F-transferase has a wide range of permissivity for its aminoacyl-substrates, it does discriminate among the natural amino acids. There is already substantial evidence that the L,F-transferase requires an unbranched beta carbon to accept an aminoacyl substrate, even if the branched amino acid is mis-charged to a methionine tRNA.[3, 9, 42] We also examined azaleucine and the phenylalanine analogs, 3,2-pyridylalanine and 3,3-pyridylalanine, all of which are charged to their respective tRNAs;[19, 43] however, we found no evidence *via* the *in vitro* assay that any of these analogs can be transferred by the L,F-transferase to the N-terminus of KA-AMC. Unsurprisingly, analogs that are not charged onto tRNA, such as pentafluorophenylalanine, are not substrates for the L,F-transferase.

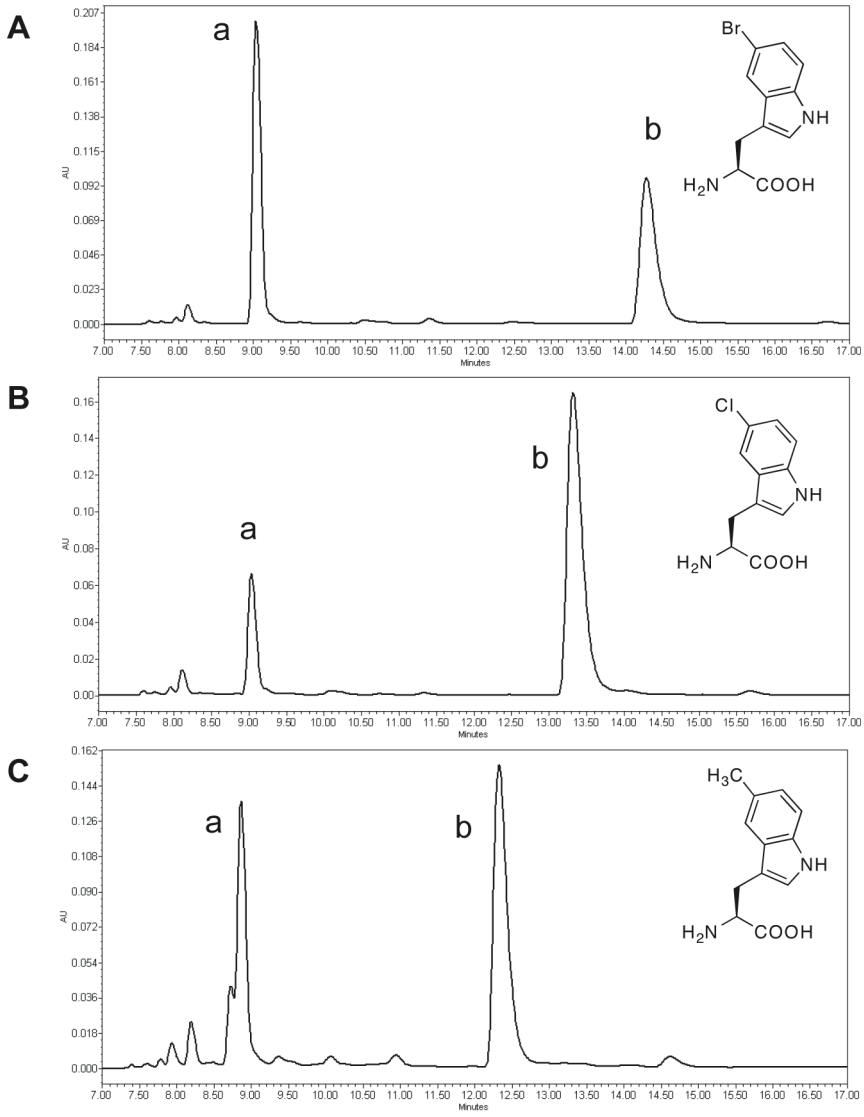


Figure 2-10. HPLC analysis of the transfer of tryptophan analogs **15**, **16**, and **17** to KA-AMC by the L,F-transferase. A) Analysis of the transfer of **15** after charging by the T415G yeast PheRS. B) Analysis of **16** after charging by the T415G yeast PheRS. C) Chromatograph showing transfer of **17** to KA-AMC after charging by the T415G yeast PheRS. In All traces, peak **a** is KA-AMC and peak **b** is the tripeptide product.

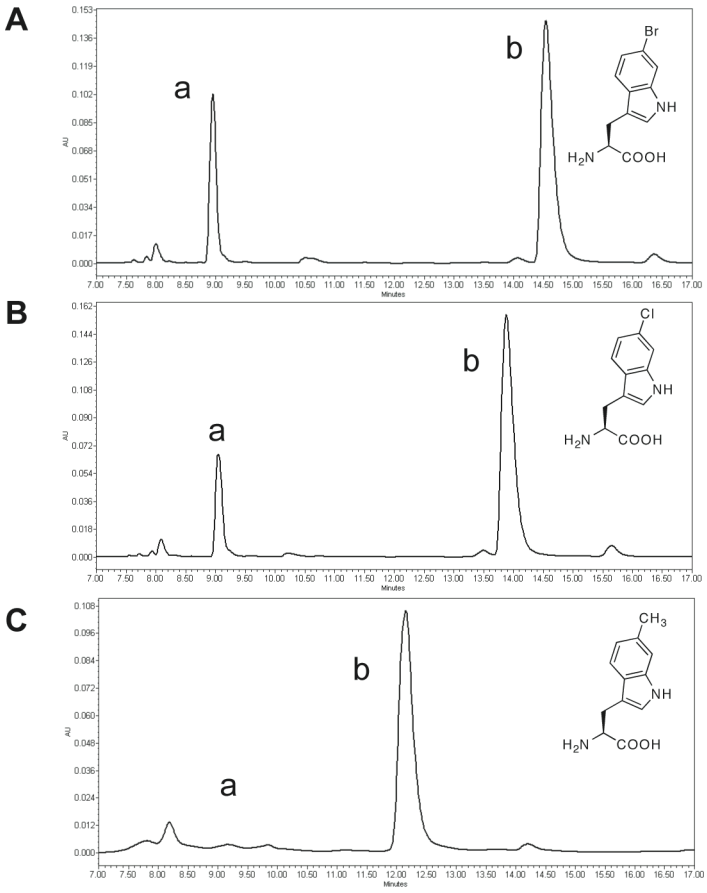


Figure 2-11. HPLC analysis of the transfer of tryptophan analogs **18**, **19**, and **20** to KA-AMC by the L,F-transferase. A) Analysis of the transfer of **18** after charging by the T415G yeast PheRS. B) Analysis of **19** after charging by the T415G yeast PheRS. C) Chromatograph showing complete transfer of **20** to KA-AMC after charging by the T415G yeast PheRS. Peak **a** is KA-AMC and peak **b** is the tripeptide product.

2.4 Conclusions

All twenty-four non-canonical amino acids shown in Scheme 2-1 were transferred to the N-terminus of KA-AMC. Under the conditions used here, analogs **2**, **4**, **5,** **7**, **8**, **9**, **19**, and **20** were transferred to the N-terminus of KA-AMC in near-quantitative yields within four hours using wild-type or mutant *E. coli* tRNA synthetases to aminoacylate the tRNA. The remaining analogs were transferred in lower yields although the yields of the coupled reactions is highly dependent on the quality of enzyme preparation. We have also shown that non-canonical amino acids are substrates for the L,F-transferase not only from *E. coli* tRNAs, but also from *S. cerevisiae* tRNA. We also identified three translationally active analogs for leucine and phenylalanine that were not substrates for the L,F-transferase.

We have shown that the *E. coli* L,F-transferase can append a wide variety of non-canonical amino acids to the N-terminus of a model dipeptide compound. These results suggest that recombinant proteins can also be N-terminally modified using *in vitro* coupled reactions. This method offers the ability to add diverse chemical functionality to peptides and proteins site-specifically. The extension of the method to the modification of proteins is explored in Chapter 3 of this thesis.

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