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Ribosomal Synthesis of N-methylated peptides

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RIBOSOMAL SYNTHESIS OF *N*-METHYLATED PEPTIDES

A thesis submitted in partial fulfillment of the requirements for the degree of

Master's of Science at Virginia Commonwealth University.

by

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I would like to thank Dr. Matthew Hartman for his support as my advisor over the last two years. As an amateur graduate student with chemistry background, he spent a lot of time to teach me biological techniques and made me familiar with chemical biology. For sure there is no one more important than him in making me interested in continuing my research life in chemical biology.

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Abstract

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Sara Ahadi, MSc.

A thesis submitted in partial fulfillment of the requirements for the degree of master's of science at Virginia Commonwealth University.

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Major Director: Matthew C T Hartman, Ph.D., Department of Chemistry

Natural peptide products isolated from various organisms often contain *N*-methylated backbones. Such a modification of backbone of the peptide changes its conformational rigidity. This modification improves the biological properties of the peptide, such as improved target affinity, proteolytic stability or membrane permeability. Therefore synthesis of *N*-methylated peptide libraries is valuable in screening for drug-like peptides suitable for therapeutic uses.

Protein synthesis using recombinant elements (PURE) and Flexizyme were used in order to reassign specific codons to *N*-methyl amino acids. mRNA-dependent translation system enable us to make our desired peptides with *N*-methyl amino acids. This technology is a convenient tool for the construction of *N*-methyl peptide libraries.

Using Flexizyme in order to make library of *N*-methyl peptides requires significant amount of tRNA. Therefore developing a simple and rapid method for purification of specific tRNA from fully modified *E. coli* total tRNA would be advantageous

Here we reported a new technique in purification of individual tRNAs using fluorous affinity tag. From total tRNA, desired tRNA could be charged with related amino acid and tagged with fluorous molecule through reductive amination.

Chapter 1

Introduction

1.1 *N*-methylated Peptides

Studies on peptide natural products show that many contain *N*-methyl amino acids[1]. This N-methylated backbone brings natural peptides special biological properties such as improved target affinity, proteolytic stability or membrane permeability[2]. This modification also alters peptides' conformational rigidity and hydrophobicity[3].

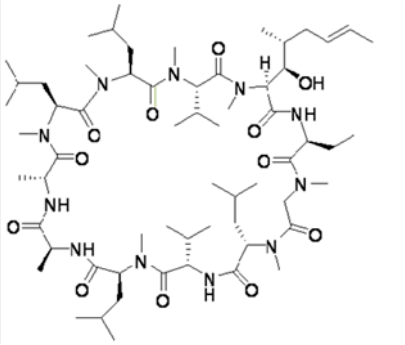


Figure 1. Cyclosporin

1.2 peptide library

A peptide library is a systematic combination of different peptides in large number. It is a powerful tool for drug discovery and structural studies. Therefore library of *N*-methylated peptides will be useful in discovery of therapeutic peptides.

There are many techniques available to generate diverse peptide libraries. Ribosome display is one of the recent techniques which is an *in vitro* selection based on the display of nascent peptide

chain on the surface of the ribosome[4, 5]. Libraries as big as 10^{12} can be made by this technique. However selections must be performed under conditions that preserve the integrity of the ribosome-mRNA-peptide complex. A more powerful in vitro selection technique is mRNA display, in which an mRNA would become directly attached to the peptide through a stable covalent link. mRNA display provides advantages over other techniques because of the potential for incorporation of unnatural amino acids as well as the ability to prepare libraries with up to 10^{13} unique peptides [6, 7].

1.3 *In vitro* Synthesis of *N*-methylated peptides

N-methyl amino acids are valuable components in preparation of *N*-methylated peptides, which have drug-like properties. The backbone *N*-methylation of these peptides are mostly executed by enzymes in multienzyme clusters, called nonribosomal peptide synthetases (NRPSs)[8, 9]. This peptide synthesis machinery is template–independent. Their complexity makes it difficult to generate desired peptide libraries[10, 11]. On the other hand, the translation machinery expresses peptides in an mRNA template-dependent manner, yet only incorporates 20 natural amino acids into peptide chain[10]. However manipulations of the translation apparatus enable us to incorporate unnatural amino acids into peptides[12]. The main technology for incorporation of unnatural amino acids is the PURE system[13], a reconstituted, *in vitro* translation consisting of purified recombinant factors from *Escherichia coli.* This system is highly suitable for translation of unnatural peptides because it provides the ability to control which amino acid and amino acid synthetases can be added to translation mix. This makes it possible to reassign multiple codons to *N*-methyl amino acids by withdrawing the corresponding natural amino acids and synthetases. Moreover this method allows us to simultaneously incorporate multiple unnatural amino acids.

Different techniques were used for incorporation of *N*-methyl amino acids into peptides. One of the most common one is amber suppression, in which an orthogonal tRNA, specific for amber stop is chemoenzymatically aminoacylated and add to cell free translation system[11-14]. A disadvantage of this technique is the inability to simultaneously incorporate two unnatural amino acids into a peptide.

1.4 Flexizyme

An alternative method in making non-natural aminoacyl tRNAs designed by Suga and coworkers, is Flexizyme[14, 15]. Flexizyme is *de novo* tRNA acylation system. It can be used for the preparation of acyl tRNAs with nearly unlimited selection of amino acids and tRNAs[16]. It works by recognizing CCA sequence at the 3` of tRNA as well as the aromatic side chain on amino acids (Figure 2).

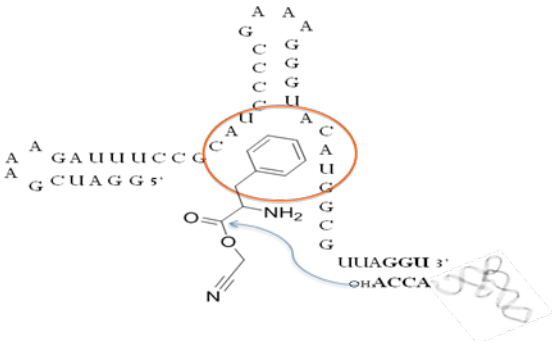


Figure 2. Flexizyme

If the amino acid has the aromatic group in its side chain, it needs to be activated by a cyanomethyl ester (CME) group. Since many amino acids lack aromatic side chains, Suga and coworkers developed a Flexizyme variant that recognizes dinitro benzyl esters (DBE) of non-aromatic amino acids[16]. This mutant Flexizyme is called dinitro-Flexizyme (dFx) has five times

better activity for dinitrobenzyl ester amino acids. The Flexizyme which recognizes aromatic amino acids is called enhanced Flexizyme (eFx).

The combination of the Flexizyme system with PURE translation apparatus allows us to perform mRNA-dependent synthesis for preparation of peptides with multiple unnatural amino acids.

Chapter 2

Flexizyme

In vitro translation of *N*-methyl peptides requires using *N*-methyl aminoacyl tRNAs in translation. In this chapter charging *N*-methyl amino acids on tRNAs is described and formation of *N*-methyl aminoacyl tRNAs is confirmed by MALDI/MS. The *N*-methyl aminoacyl tRNAs were used in translation which is discussed in chapter 3.

2.1 Flexizyme

Flexizyme is a ribozyme which catalyzes tRNA acylation. It has been developed by Suga and coworkers as an alternative method to make non-natural aminoacyl tRNAs[15]. Flexizyme can be used to charge any amino acid on any tRNA. It works by recognizing CCA sequence at the 3` end of tRNA as well as the aromatic side chain on amino acids. There are two types of Flexizyme: eFx and dFx. eFx can be used for amino acids with aromatic side chains that are activated by cyanomethyl ester group. However, dFx is used for amino acids without aromatic side chains and activated with dinitrobenzyl ester group.

2.2 *N*-methyl amino acids

Six different *N*-methyl amino acids were chosen for the study: *N*-methyl alanine, *N*-methyl serine, *N*-methyl glycine, *N*-methyl phenylalanine, *N*-methyl *p*-nitro-phenylalanine and *N*-methyl tyrosine (Figure 3). These *N*-methyl amino acids have been reported to be incorporated well into peptides by Flexizyme combined with in vitro translation[2]. Among these amino acids, *N*-methyl phenylalanine, *N*-methyl *p*-nitro phenylalanine and *N*-methyl tyrosine have aromatic side chains and must be activated via the cyanomethyl ester derivative. For the other three amino acids

without aromatic side chains, the dinitrobenzyl ester group is needed for activation and recognition by Flexizyme.

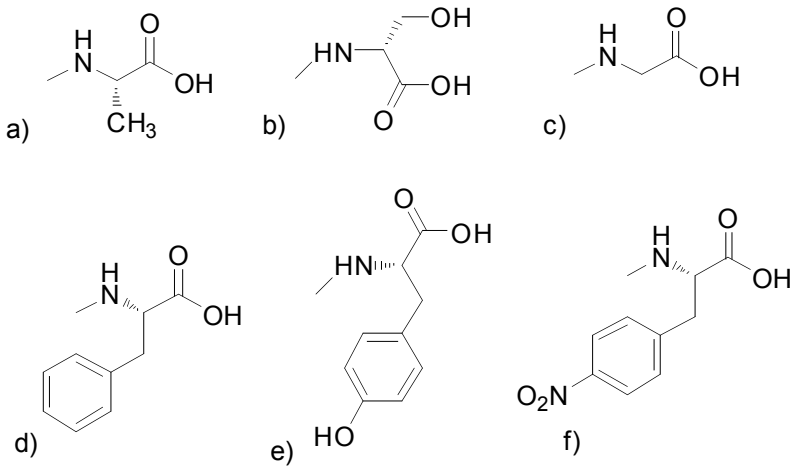


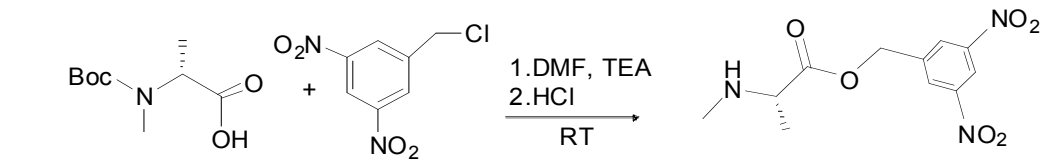
Figure 3. Structure of *N*-methyl amino acids a) *N*-methyl alanine, b) *N*-methyl serine, c) *N*-methyl glycine, d) *N*-methyl phenylalanine, e) *N*-methyl tyrosine, f) *N*-methyl *p*-nitro-phenylalanine

2.3 Synthesis of DBE substrates

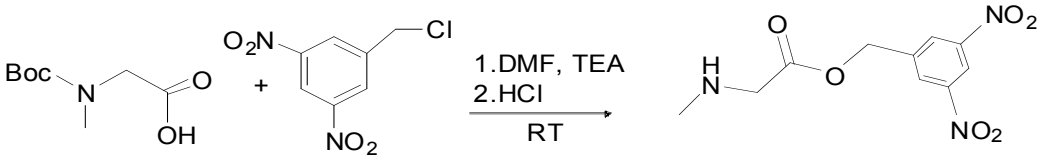
In order to synthesize DBE substrates, Boc-protected *N*-methyl amino acids were used. The reaction occurs in presence of a base, triethylamine (TEA), which deprotonates the carboxylic acid group. The resulting nucleophile can attack 3,5-dinitrobenzyl chloride forming the ester bond. The reaction mixture was stirred in dimethylformamide at room temperature for 12 hours.

2.3.1 Dinitrobenzyl ester substrate synthesis

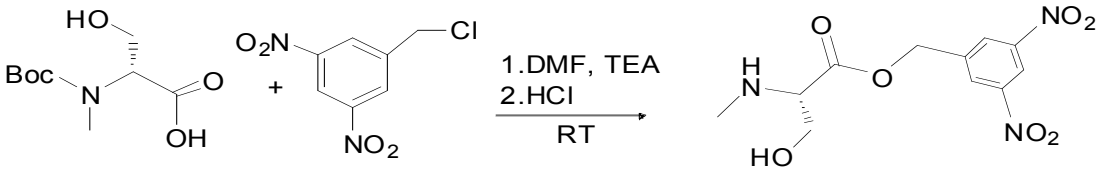
Formation of DBE substrates was confirmed by ^{1}H NMR.



Yield: 76%



Yield: 64%

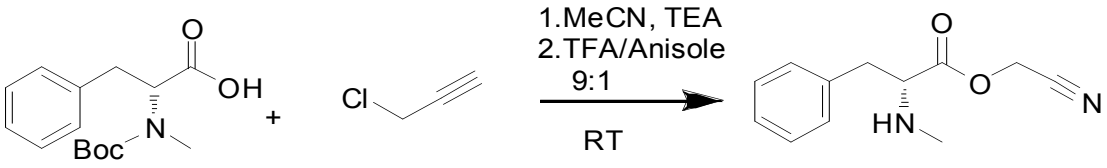


Yield: 65%

Scheme 1. Synthesis of DBE substrate of alanine, glycine and serine

2.3.2 Cyanomethyl ester substrate synthesis

Formation of CME substrates was confirmed by ^{1}H NMR.



Yield: 57%

Scheme 2. Synthesis of CME substrate of phenylalanine

2.4 tRNA

In order to facilitate synthesis of peptide libraries, tRNAs should be orthogonal and able to incorporate an *N*-methyl amino acid at a single template-directed position in a polypeptide without significant competition from other amino acids present in the translation mixture. $tRNA^{Asn\text{-}E2}$ (NNN) is a tRNA which the Flexizyme system is able to efficiently charge unnatural amino acids onto (Figure 4)[17].

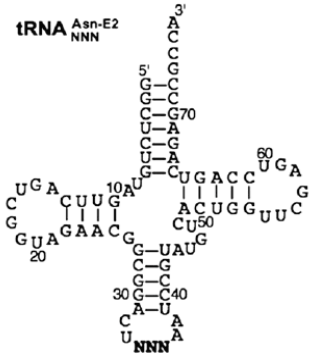


Figure 4. $tRNA^{Asn\text{-}E2}$ (NNN)

Six different tRNAs bearing unique anticodons (NNN) were chosen for this work (Table 1). These anticodons can replace amino acids for codons that can be read by only a single tRNA. This is important in order to assign a unique codon to each *N*-methyl amino acid in peptide library. Codons ending in C or G were preferred because they make tighter interactions with complimentary bases (Figure 5).

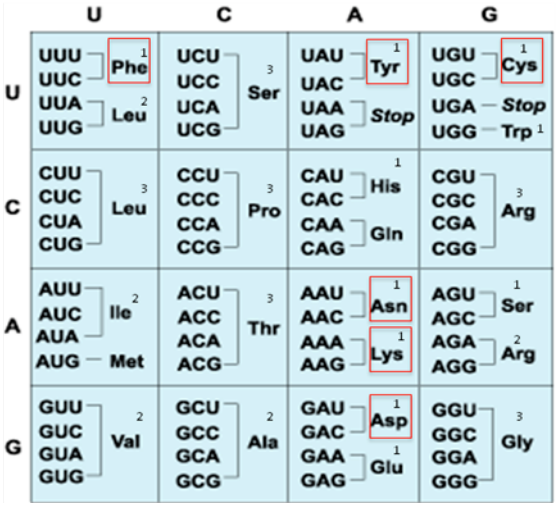


Figure 5. Table of amino acid codons. Codons of amino acids in squares were chosen for library

Table 1. tRNA/amino acid pairs

$tRNA^{Asn\text{-}E2}$ (NNN)	Codon	Natural amino acid	Unnatural amino acid
$tRNA^{Asn\text{-}E2}$ (GUC)	GAC	Aspartic acid	*N*-Me-Alanine
$tRNA^{Asn\text{-}E2}$ (GUU)	AAC	Asparagine	*N*-Me-Serine
$tRNA^{Asn\text{-}E2}$ (GUA)	UAC	Tyrosine	*N*-Me-Tyrosine
$tRNA^{Asn\text{-}E2}$ (GAA)	UUC	Phenylalanine	*N*-Me-Phenylalanine
$tRNA^{Asn\text{-}E2}$ (CUU)	AAG	Lysine	*N*-Me-*p*-nitro phenylalanine
$tRNA^{Asn\text{-}E2}$ (GCU)	AGC	Cysteine	*N*-Me-Glycine

2.4.1 Synthesis of tRNA

These tRNAs were transcribed from their synthesized DNA sequences using T7 RNA polymerase. To prepare the templates, four different primers were synthesized, annealed together and amplified by PCR (figure 6). Table 2 shows the sequence of these four primers. Primer B and A were first amplified in PCR and then primer C which contains the T7 promoter sequence and D were added to be annealed to main template and make desired double strand DNA. Primer B is part of the tRNA, which has an anticodon sequence and therefore is different in each of the tRNAs. The DNA product was transcribed by using T7 polymerase and purified by 10% denaturing PAGE.

Figure 6. Sequence of primers used in making DNA templates

Table2. Sequence of DNA primers for each tRNA

Primer A	5'-GTAAT ACGAC TCACT ATAGG CTCTG TAGTT CAGTC GGTAG AACGG CGGA-3'

Primer C	5'-GGCGT AATAC GACTC ACTAT AG-3'

Primer D	5’-TGGCG GCTCT GACTG GACTC GAACC AGTGA CATAC GGA-3’

$tRNA^{Asn-E2}$ (NNN)	Primer B
$tRNA^{Asn-E2}$ (GUC)	5’-GAACC AGTGA CATAC GGACT GACAA TCCGC CGTTC TACCG ACT-3’
$tRNA^{Asn-E2}$ (GUU)	5’-GAACC AGTGA CATAC GGACT AACAA TCCGC CGTTC TACCG ACT-3’
$tRNA^{Asn-E2}$ (GUA)	5’-GAACC AGTGA CATAC GGACT TACAA TCCGC CGTTC TACCG ACT-3’
$tRNA^{Asn-E2}$ (GAA)	5’-GAACC AGTGA CATAC GGACT TTCAA TCCGC CGTTC TACCG ACT-3’
$tRNA^{Asn-E2}$ (CUU)	5’-GAACC AGTGA CATAC GGACT AAGAA TCCGC CGTTC TACCG ACT-3’
$tRNA^{Asn-E2}$ (GCU)	5’-GAACC AGTGA CATAC GGACT TGCAA TCCGC CGTTC TACCG ACT-3’

2.4.2 PCR troubleshooting

As described in section 2.4.1, DNAs sequencing desired tRNAs were made by PCR. To obtain the best PCR product main templates (A and B) were first amplified in two cycles by using Taq DNA enzyme. Then T7 Forward primer (Primer C) and primer D were added. After that PCR were run for 26 cycles and after 8^{th} cycle, time points were taken every other cycle. Data from time points showed us that 18 cycles is enough to get saturated PCR product. The concentration of primers was optimized.

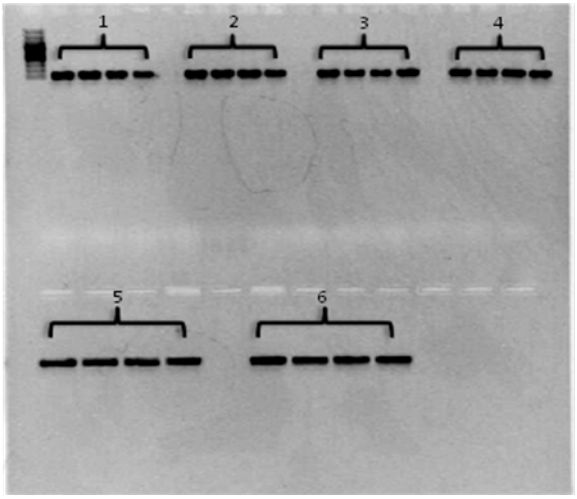


Figure 7. PCR products of six DNA templates on 2% agarose gel; DNA: 1)GAC 2)AAC 3)UAC 4)UUC 5)AAG 6)UGC

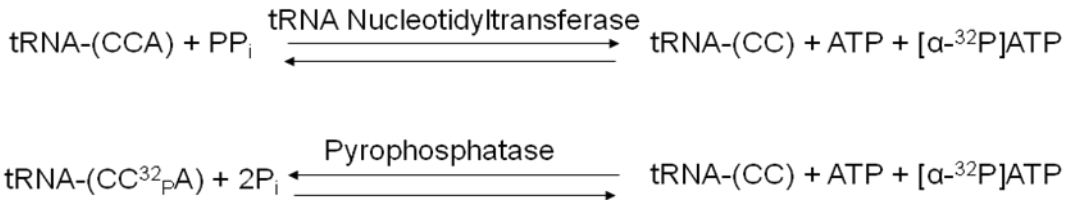
2.5 Flexizyme Reaction

To measure the time needed for acylation of *N*-methyl amino acids by Flexizyme and to make sure that the correct aminoacyl-tRNA is made, we used a ^{32}P and MALDI-TOF charging assay described in next sections.

2.6 Labling tRNA with ^{32}P

A sensitive assay for aminoacylation of tRNA is the tRNA internucleotide labeling strategy described by Uhlenbeck and coworkers[18]. The assay employs a 3`-$^{32}P$-labled tRNA prepared by an exchange reaction with [α-$^{32}P$] ATP and $PP_i$ catalyzed by *E. coli* tRNA terminal nucleotidyl transferase (scheme 3). The resulting tRNA has $^{32}P$ at between the 3` terminal C and A. During the assay, the C-terminal A is aminoacylated. Aliquots from the reaction are removed at various times and are quenched with nuclease P1 solution in acetate buffer at pH 5.0. This digestion

provides radiolabeled ^{32}P-AMP and aminoacyl-AMP which reflects the amount of aminoacylated and nonacylated tRNA at certain time point. These two radioactive products were separated by PEI-TLC and their ratio measured by a phosphorimager. Figure 8 shows TLC analysis of the aminoacylation of *N*-methyl serine, *N*-methyl glycine and *N*-methyl alanine onto tRNA^{Val}.



Scheme 3. Labeling tRNA with ^{32}P

From this ^{32}P assay data, the time point which gave the maximum yield was determined. The data for *N*-methyl amino acids is close to the literature report[19] of aminoacylation of serine (4 hr), glycine (2 hr), alanine (2 hr) acids with Flexizyme (Figure 9a, 9b and 9c).

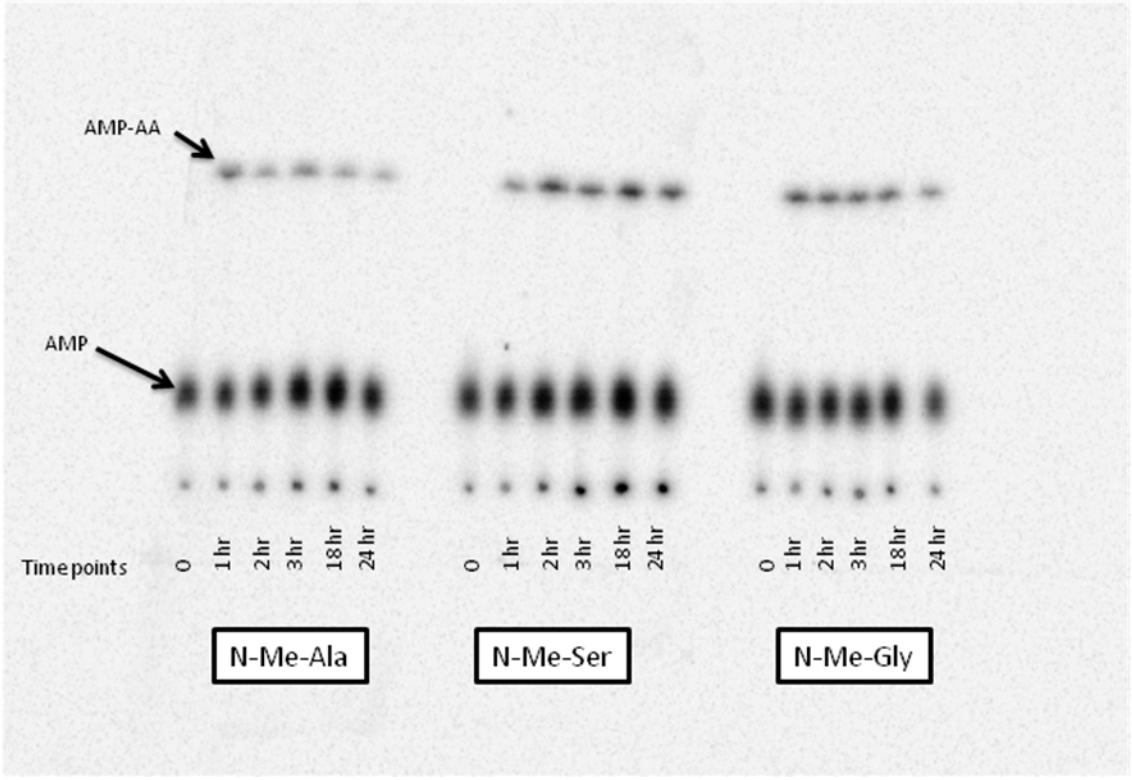
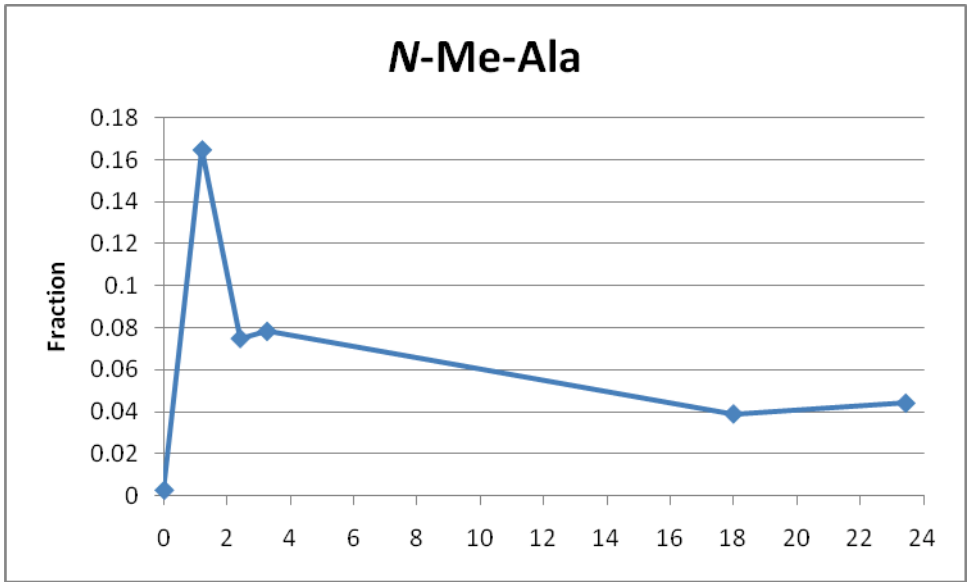
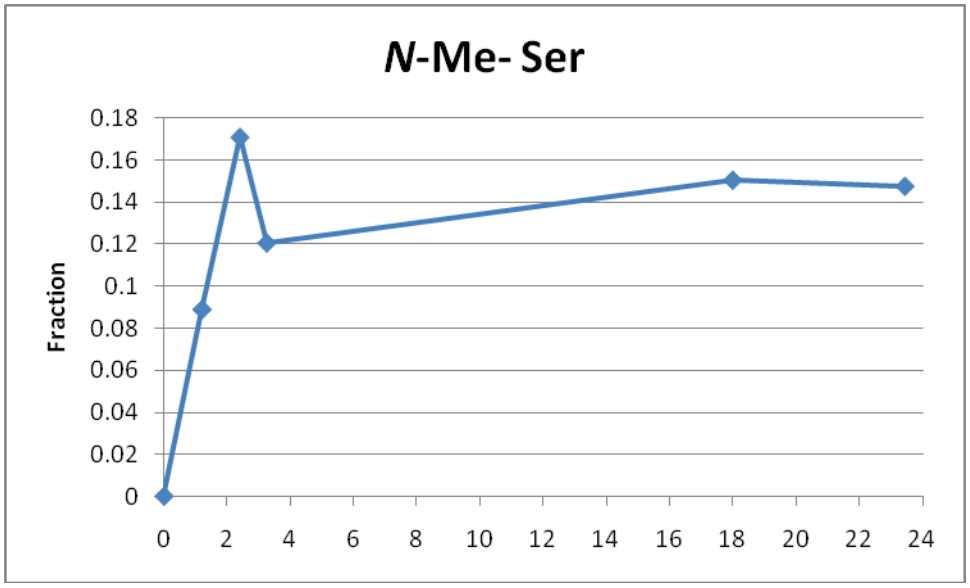


Figure 8. TLC of ^{32}P assay on *N*-methyl alanine, *N*-methyl serine and *N*-methyl glycine

a)



b)



c)

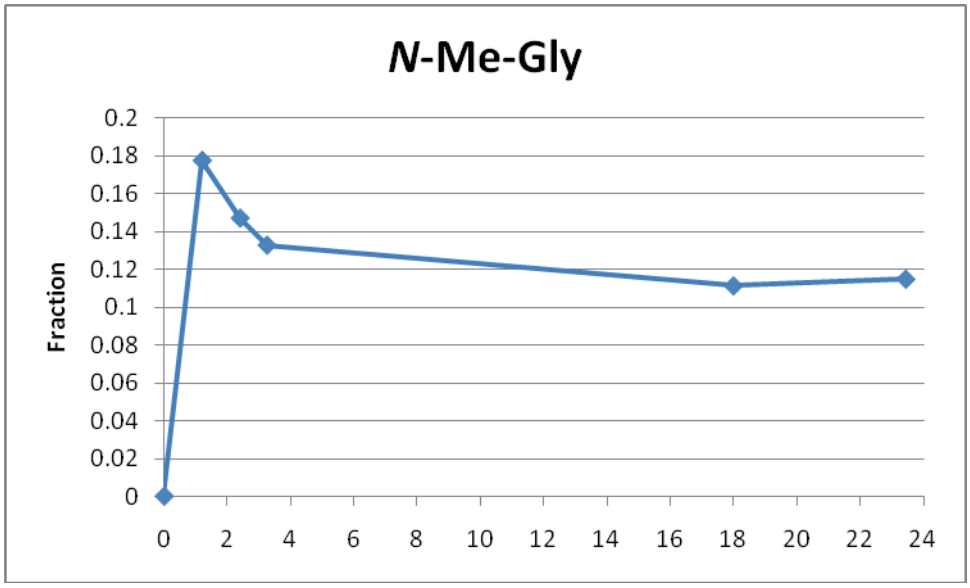


Figure 9. Aminoacylation of *N*-methyl amino acids at different time points. (Y axis shows the fraction of Amp-AA and free Amp)

2.7 tRNA aminoacylation assay based on MALDI/MS

An assay described by Szostak and coworkers[20], based on MS was chosen to verify that the correct AA-tRNA product was formed. Using MS for direct detection of the large polyionic AA-tRNA has low sensitivity, and it is unable to resolve the small mass differences between analogs. This assay solves these problems by cleaving the AA-tRNA into mononucleotides. To have higher mass in MALDI-MS, AA-tRNA was derivatized on the primary amine of the amino acid using reductive amination between the amine and aldehyde-containing triphenylphosphonium group. After derivatization the tRNA is digested with nuclease P1 at pH 5.0. MALDI-MS analysis of the derivatized AA-AMP identifies the amino acid that has been charged. In all mass spectra a peak corresponding derivatized AMP at mass 756.23 was observed.

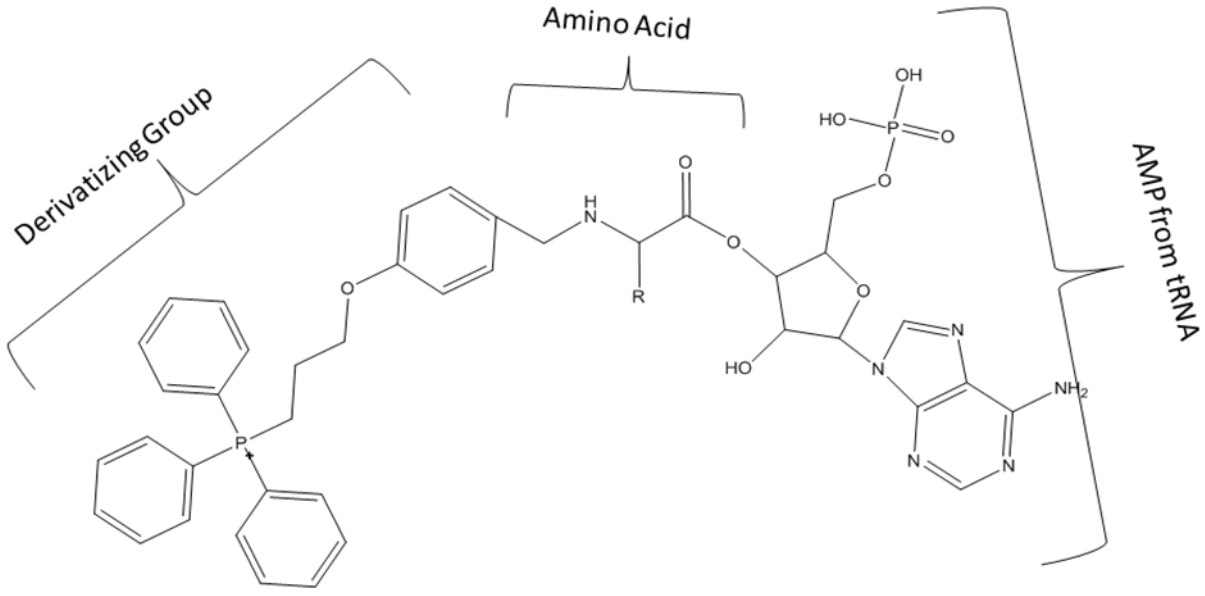


Figure 10. Derivatized AMP with phosphonium-containing benzaldehyde (2' and 3' of the tRNA, both can be acylated with the amino acid)

2.7.1 Aminoacylation of *N*-methyl amino acids on tRNA $^{Asn-E2}$

N-methyl alanine, *N*-methyl glycine, *N*-methyl serine and *N*-methyl phenylalanine were charged on tRNA $^{Asn-E2}$(GCU).The formation of aminoacyl-tRNAs were confirmed by MALDI/MS.

Table 3. Mass of aminoacyl-tRNAs

Aminoacyl tRNA	Calculated Mass	Observed Mass
N-Me-Ser-Amp	858.212	857.343
N-Me-Ala-Amp	841.332	842.338
N-Me-Gly-Amp	827.431	828.31
N-Me-Phe-Amp	917.311	918.115

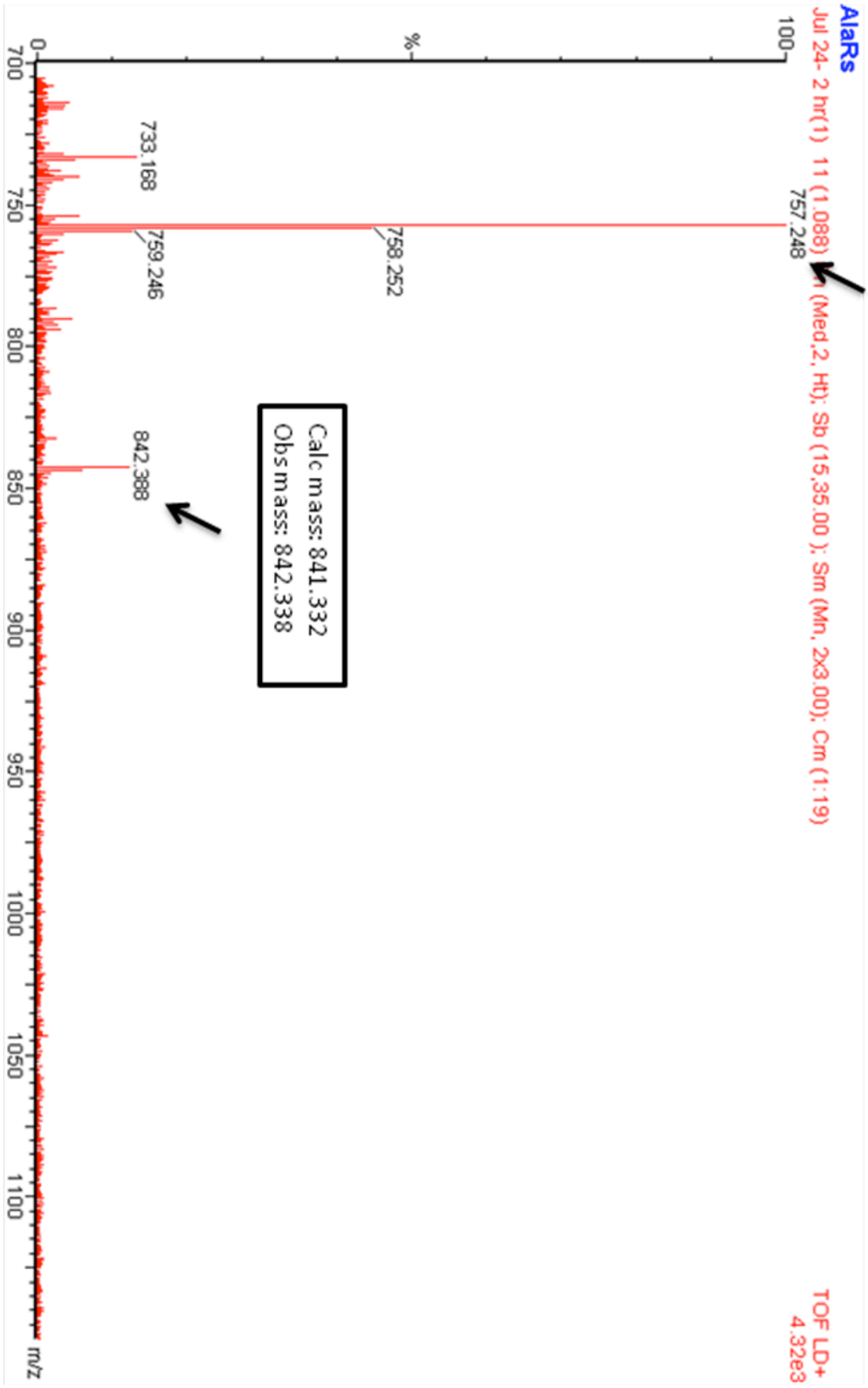


Figure 11. MALDI/MS analysis of charged *N*-methyl alanine

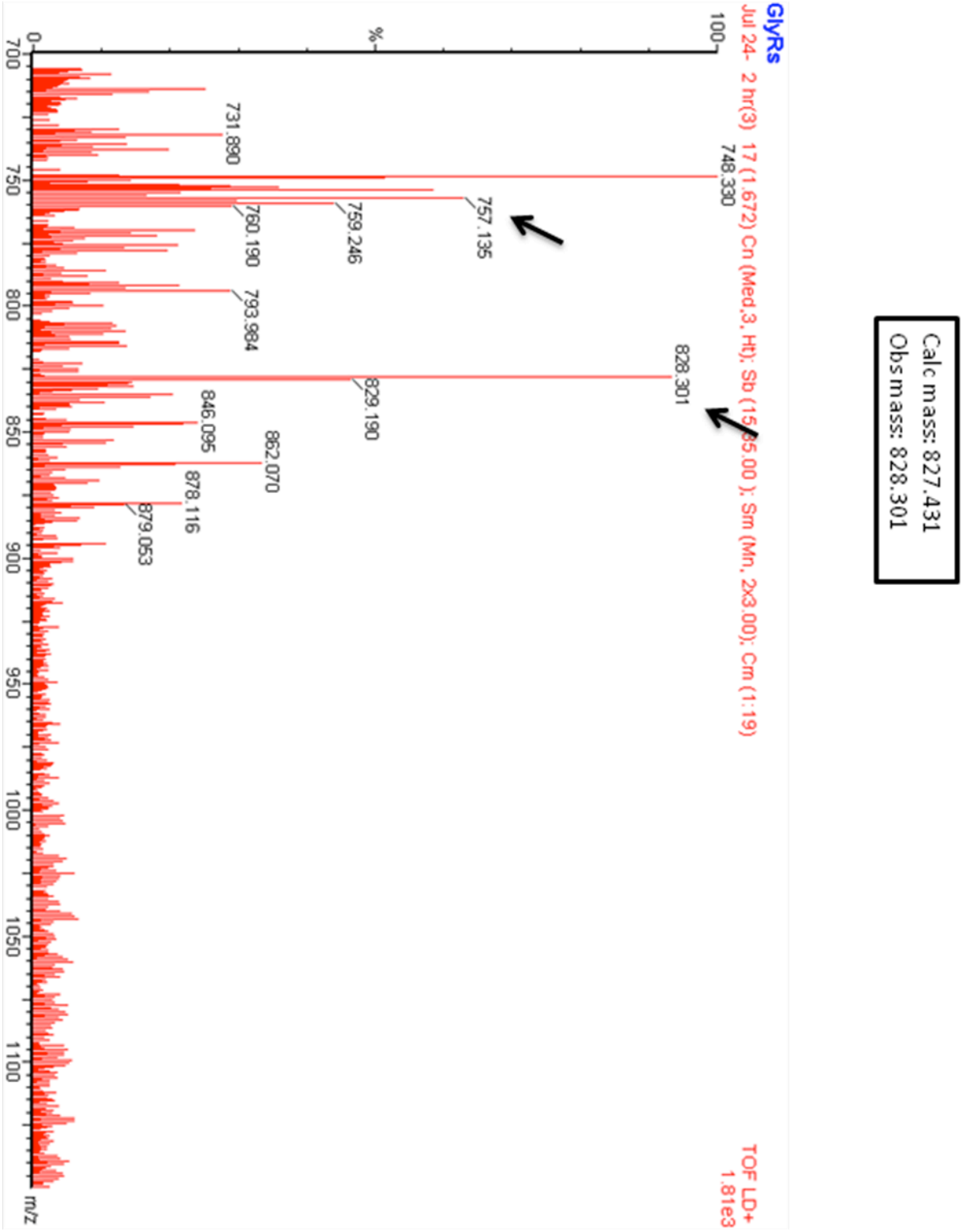


Figure 12. MALDI/MS analysis of charged *N*-methyl glycine

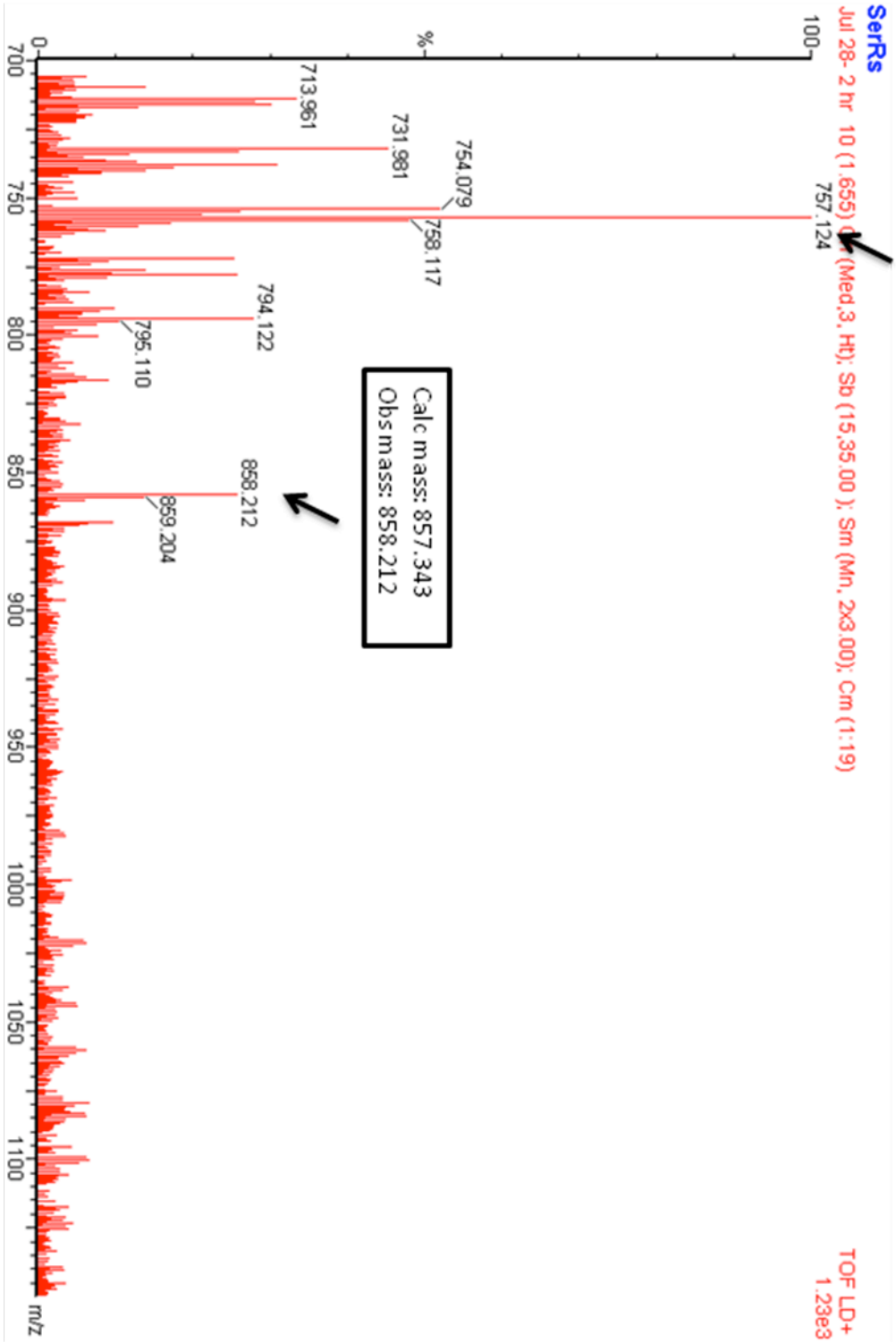


Figure 13. MALDI/MS analysis of charged *N*-methyl serine

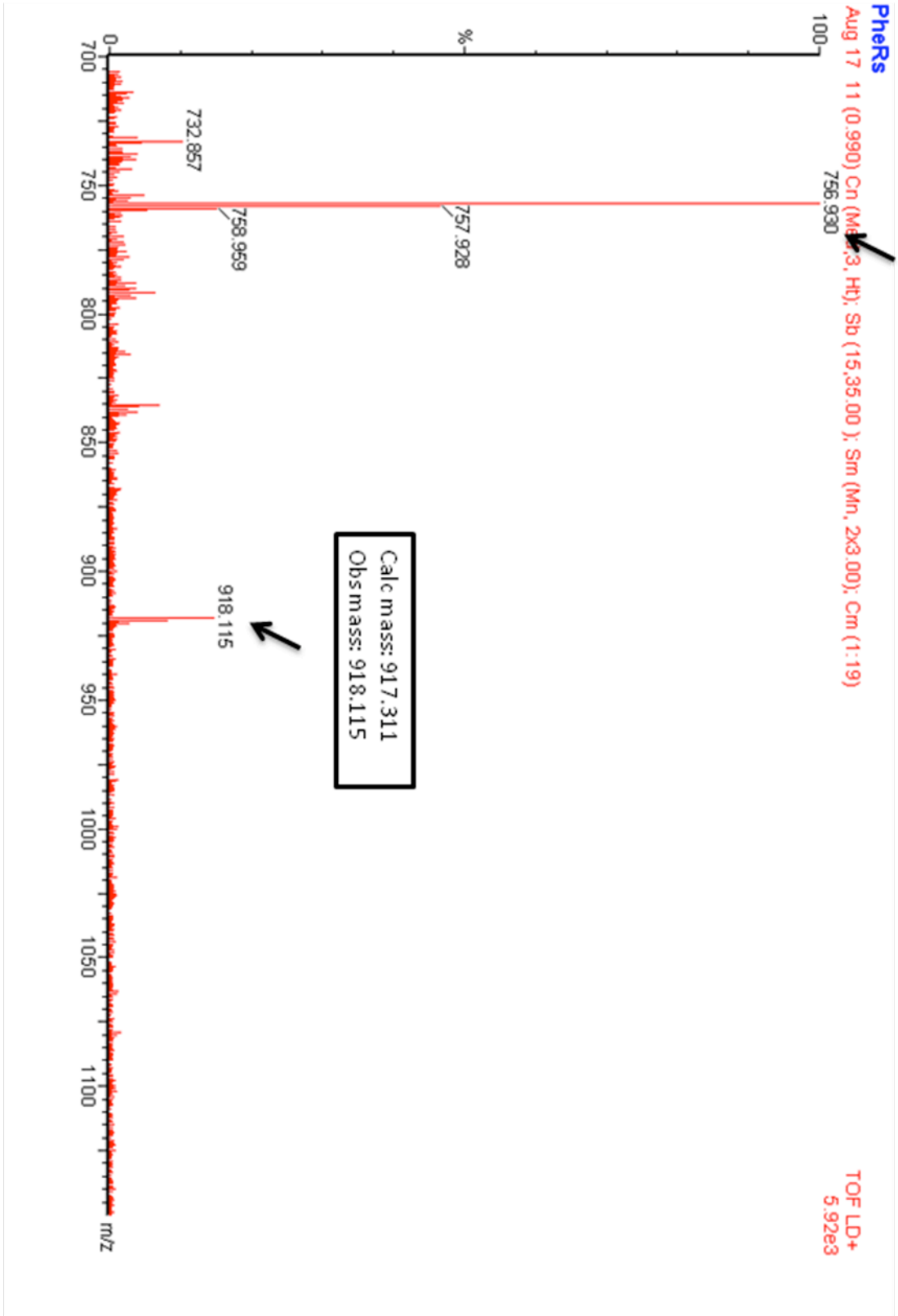


Figure 14. MALDI/MS analysis of charged *N*-methyl serine

2.8 Experimental section

2.8.1 General methods

All N-methyl amino acids were purchased from Chem Impex. Chloroacetonitrile was purchased from Sigma. All solvents (triethylamine, acetonitrile and dimethylformamide) were distilled prior to use in reaction.

MALDI mass spectra were recorded using Micromass' M@LDI (MALDI TOF) micro channel plate spectrometer. 1 μL of sample was mixed with 9 μL of MALDI matrix (1% w/v CHCA in 1:1 MeCN: 0.1% trifluoroacetic acid) and 1 μL was spotted on MALDI plate. Mass scans were obtained in the range of 700-1200 m/z.

^{1}HNMR was recorded on Bruker Avance 400 MHz spectrometer in DMSO-d_6 All signals are reported in ppm with the residual DMSO peak assigned to 2.50. The data is reported as: chemical shifts (ppm), multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet) and integration.

The DNA templates of tRNAs were made on MerMade 4 DNA synthesizer. Synthesized DNAs were deprotected with methylamine/ammonia mix solution and purified with 10% denaturing PAGE.

^{32}P PEI TLC plates were analyzed using a Typhoon 9410 phosphoimager from GE Healthcare Company.

2.8.2 Synthesis of N-methyl alanine dinitrobenzyl ester[2]

N-Boc-methyl alanine (121.92 mg, 0.6 mmol) was dissolved in DMF (100 μL). Triethylamine (138 μL, 1 mmol) and 3',5'-dinitrobenzyl chloride (108 mg, 0.5 mmol) were added and the

reaction was stirred at room temperature overnight. After the reaction was completed, liquid-liquid extraction was done with diethyl ether (9 mL) and the solution was washed with of 0.5 M HCl (3 mL x 3), 4% $NaHCO_3$ (3mL x 3) and brine (5mL). The ether layer was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was dissolved in 2 mL of 1:3 HCl:ethylacetate and incubated for 20 min at room temperature. The solution was concentrated under vacuum and the product was precipitated by the addition of diethylether (3 mL). The solid was filtered leaving 130 mg of a white powder (76%). ^{1}H NMR (DMSO-d_6, 400MHz) δ 9.35 (s, 1H), 8.83 (s, 1H), 8.70 (s, 2H), 5.56 (s, 2H), 4.23 (q, 1H), 2.6 (s, 3H), 1.5 (d, 3H).

2.8.3 Synthesis of N-methyl serine dinitrobenzyl ester

N-Boc-methyl serine was prepared according to the procedure described for *N*-methyl alanine. The solid was filtered leaving white powder (64%). ^{1}H NMR (DMSO-d_6, 400MHz) δ 9.36 (d, 1H), 8.86 (s, 2H), 8.75 (s, 1H), 5.54 (s, 2H), 4.31 (d, 2H) 3.97 (q, 1H), 2.6 (s, 3H), 1.5 (d, 3H).

2.8.4 Synthesis of N-methyl glycine didnitrobenzyl ester

N-Boc-methyl glycine was prepared according to the procedure described for *N*-methyl alanine. The solid was filtered leaving white powder (65%). ^{1}H NMR (DMSO-d_6, 400MHz) δ 9.36 (s, 1H), 8.80 (s, 2H), 8.72 (s, 1H), 5.41 (s, 2H), 4.08 (q, 1H), 2.57 (s, 3H).

2.8.5 Synthesis of N-methyl phenylalanine cyanomethyl ester[21]

N-Boc-methyl phenylalanine (800 mg, 2.86 mmol) was dissolved in 2.85 mL acetonitrile. 2.85 mL of triethylamine was added and the reaction mixture was cooled to 0 °C. Chloroacetonitrile (361 μL, 5.72 mmol, 2 equiv) was added. The reaction was warmed to room temperature and

stirred overnight. Ethyl acetate (30 mL) was added to the solution and reaction mixture was extracted with 0.5 M HCl (30 mL). The organic layer was washed with brine (30 mL), dried over $MgSO_4$ and concentrated under vacuum leaving yellow oil containing Boc-Phe-CME.

Boc-Phe-CME was deprotected by 9:1 trifluoroacetic acid (TFA)/ anisole (500 µL) under argon for 30 min. The solvent was removed under vacuum and 4M HCl was added. The solvent was removed by vacuum and the product was precipitated by addition of anhydrous ether. The precipitate was dissolved in minimal amount of methanol and precipitated again by anhydrous ether leaving a powder (57%). ^{1}H NMR (DMSO-d_6, 400MHz) δ 7.24-7.34 (m, 5H), 5.10 (s, 2H), 4.42 (t, 1H), 3.07-3.21 (m, 2H), 2.6 (s, 3H).

2.8.6 Preparation of $tRNA^{Asn-E2}$ (NNN) and flexizyme

For the preparation of the DNA template for $tRNA^{Asn-E2}$, primer A and B (0.5 µM each) were amplified with Taq DNA polymerase and then primer C and D were annealed to main template and extended. The resulted double strand DNA was transcribed in presence of T7 polymerase[22] and purified by 10% denaturing PAGE.

Following DNA templates were used in preparation of dFx and eFx[19]. They are then transcribed to Flexizyme in presence of T7 polymerase.

dFx: 5'-TGGTACCTAACGCCATGTACCCTTTCGGGGATGCGGAAATCTTTCGATCC-3'

eFx: 5'-TGGTACCTAACGCTAATCCCCTTTCGGGGCCGCGGAAATCTTTCGATCC-3'

T-7 Primer: 5'-TAA TAC GAC TCA CTA TAG GG-3'

2.8.7 Flexizyme reaction

$tRNA^{Asn\text{-}E2}$ (NNN) was first folded under the following conditions: 40 μM tRNA in 0.2 M HEPES-KOH buffer (pH 7.5) was heated in a PCR machine at 95 °C for 1 min followed by cooling to room temperature gradually over 5 min. 10 μL of 3M $MgCl_2$ and 5 μL of 200μM dFx or eFx were added to the solution. After 5 min, 10 μL of 25mM N-methylated amino acid substrate (DBE or CME) in DMSO was added to the mixture and incubated on ice for 1-4 hours. The acylation was quenched by addition of 150 μL 0.6 M NaOAc and the aminoacyl-tRNA was recovered after precipitation with 3 volumes of ethanol.

2.8.8 Labeling tRNA with ^{32}P

tRNAs ^{32}P-labled at 3`-terminus were prepared by an ATP-PPi exchange reaction catalyzed by *E. coli* tRNA terminal nucleotidyl transferase. 20 mM Na Tris/$MgCl_2$ pH 8.7, 1 μM tRNA, 0.17 μM [α-^{32}P] ATP (10 mCi/ml), 500 μM sodium pyrophosphate and tRNA nucleotidyltransferase (30 μg/ml) were added together and incubated in 37°C for 2 hours. The reaction was quenched with 10 μL inorganic pyrophosphatase (10 units/mL) and kept at 37°C for 2 min. The solution was extracted with phenol:$CHCl_3$:isoamyl alcohol 25:24:1. The 32P-labled tRNA was passed through a NAP-5 column to be desalted and purified from unincorporated nucleotides. tRNA was recovered by YM-10 centrifugal filter units with membrane with 10000 molecular weight cut off and counted on a scintillation counter. 200000 cpm of this labeled-tRNA was used in the Flexizyme reaction, and the final conversion to aminoacyl tRNA was measured by determining the ratio between AA-AMP and AMP spots on a PEI-TLC plate. Samples were kept on ice for

aminoacylation. 1μL of sample was withdrawn at desired time points (0, 1, 2, 4, 18 and 24 hours) and mixed with 4μL of 200mM NaOAc, pH 5.0 containing 1unit/μL nuclease P1. The digest proceeded for 10 min at room temperature. Approximately 1μL of this sample was spotted on polyethylenimine-cellulose plates. The plates were pre-run with water and eluted with Glacial acetic acid and/ 1M NH_4Cl/H_2O (5:10:85). The intensity of the AA and AA-AMP spots were analyzed using a phorphorimager.

2.8.9 tRNA aminoacylation assay based on MALDI/MS

Aminoacyl tRNA prepared using the Flexizyme reaction was dissolved in 200 mM NaOAc (pH 5.0, 12.5 μL). Half of the solution was stored at -20°C and the remaining half was added to water (3.75 μL) and freshly prepared 4-formylphenoxypropyl triphenyl phosphonium bromide (69 mM, 12.5 μL) in MeOH. At the last step freshly prepared $NaCNBH_3$ (200mM, 2.5 μL) dissolved in 50mM NaOAc, pH 5.0 was added and a white solid precipitated but then redissolved upon heating. The solution was incubated in 37°C for 2 hours using a tumbler. The reaction was quenched by 4.4 M NH_4OAc (pH 5.0, 2.5 μL) and the RNA was precipitated with 100% EtOH (75 μL). The solution was stored in -20°C for 20 min and centrifuged at high speed for 20 min at 4°C. The pellet was washed with 70% EtOH (2X) and 100% EtOH (2x). The pellet was air dried for 5-10 min and then dissolved in 200 mM NH_4OAc, pH 5.0 (2.25 μL) and 0.25 μL of 1 unit/μL nuclease P1 was added. After 20 min the reaction was quenched on ice and 1 μL of the solution was mixed with 9 μL of MALDI matrix (1% CHCA in 1:1 MeCN: 0.1% trifluoroacetic acid). The solution was vortexed and 1 μL was spotted on MALDI plate for analysis.

2.9 Discussion

N-methyl amino acid substrates (CME or DBE) were synthesized. DNA templates for tRNAs were synthesized and amplified in PCR. PCR troubleshouting was done to obtain a single band from PCR products.

The flexizyme system was used to charge *N*-methyl amino acids on $tRNA^{Asn\text{-}E1}$ (NNN). The formation of aminoacyl-tRNA was confirmed using a MALDI-TOF charging assay and the percentage of charging was measured by using ^{32}P labeled tRNA. The prepared aminoacyl-tRNA is ready for use in the PURE translation system to create *N*-methylated peptides (Chapter 3).

Chapter 3

Translation

In this chapter, incorporation of N-methyl amino acids into peptide was studied *in vitro*. *N*-methyl amino acids were assigned for natural codons in PURE translation where N-methyl aminoacyl tRNAs were added. Formation of N-methylated peptides was confirmed with MALDI/MS.

3. 1 PURE Translation

PURE – protein synthesis using recombinant elements- is a reconstituted *E. coli* cell-free translation system[13]. The most important feature of this translation system is that some components, such as amino acids and aminoacyl-tRNA synthetases (aaRSs), can be withdrawn from translation mixture and therefore leave vacant specific codons. Later one can reassign the vacant codons to a desired unnatural amino acid to create unnatural peptides as programmed by a particular mRNA sequence.

3.2 mRNA

In this work an mRNA was designed to express a peptide with N-terminal His tag which was followed by eight different amino acids: Met-Phe-Asp-Asn-Tyr-Lys-Cys-Arg. In this sequence the desired *N*-methyl amino acids could be substituted instead of each of the natural ones. To

find an mRNA that could be transcribed and translated well, three different sequences of mRNA with same amino acid codons were designed and their efficiency in translation was compared to each other (Table 4).

Table 4. Three mRNAs

mRNA-1	AUG CAC CAU CAC CAU CAC CAU AUG TTC GAC AAC TAC AAG TGC AGG UAG UAG
(Peptide)	MHHHHHHMFDNYKCR
mRNA-2	AUG CAC CAU CAC CAU CAC CAU AUG GAC TTC AAGAAC TGC TAC AGG UAG UAG
(Peptide)	MHHHHHHMDFKNCYR
mRNA-3	AUG CAC CAU CAC CAU CAC CAU AUG AAG AAC TGC TAC TTC GAC AGG UAG UAG
(Peptide)	MHHHHHHMKNCYFDR

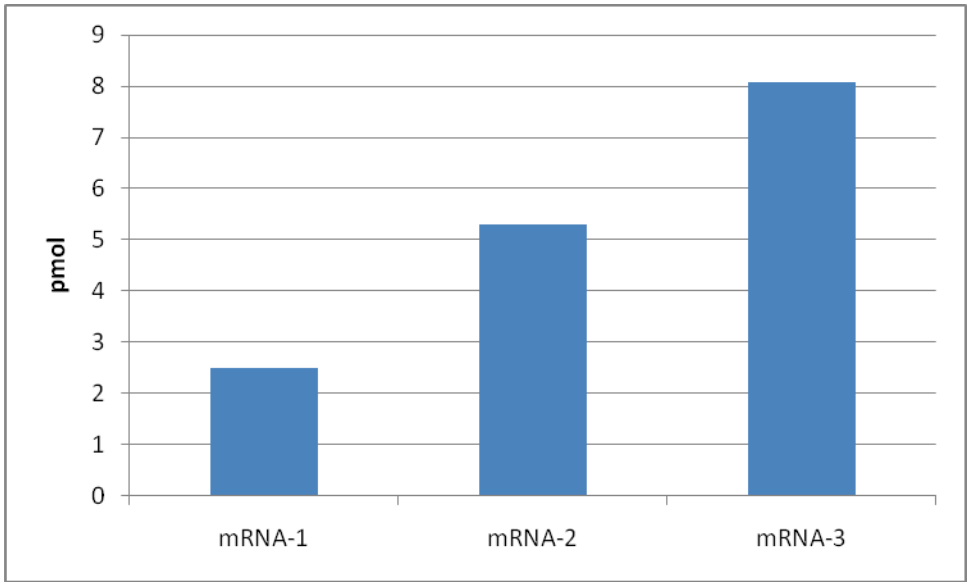


Figure 15. Translation yields of three mRNAs

mRNA-3 was chosen to be used in translations because it gave the highest yield.

3.3 Incorporation of *N*-methyl amino acids in peptide

PURE translation system was used for single incorporation of *N*-methyl glycine, *N*-methyl alanine and *N*-methyl phenylalanine into peptide backbones. *N*-methyl amino acids were charged on tRNAs by the Flexizyme system (chapter 2) and were used in PURE translation. In choosing pairs of tRNA and amino acids, we tried to assign each natural amino acid codon to the same *N*-methyl amino acid to maintain the side chain diversity in our peptide library. We selected a Phe codon (UUC) to assign to *N*-methyl phenylalanine charged on orthogonal $tRNA^{Asn\text{-}E2}$. Cys codon (UGC) was selected for *N*-methyl glycine and Asn codon (AAC) was selected for *N*-methyl alanine. Figure 16 shows the yield of 50 µL translation for these three N-methyl amino acids.

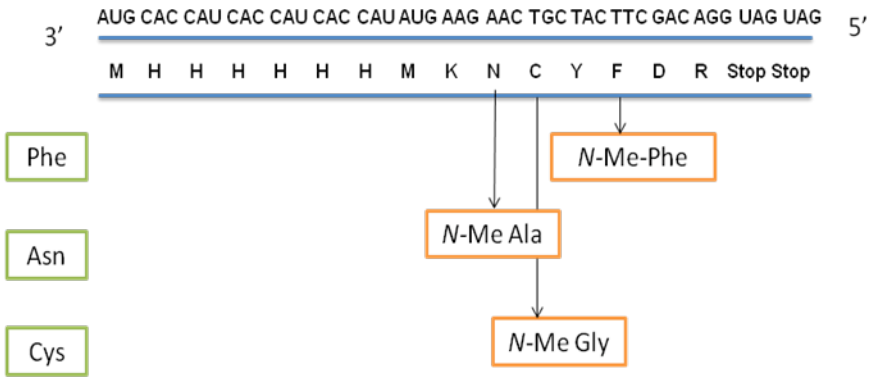


Figure 16. *In vitro* translation of *N*-methyl alanine, *N*-methyl phenylalanine and *N*-methyl glycine

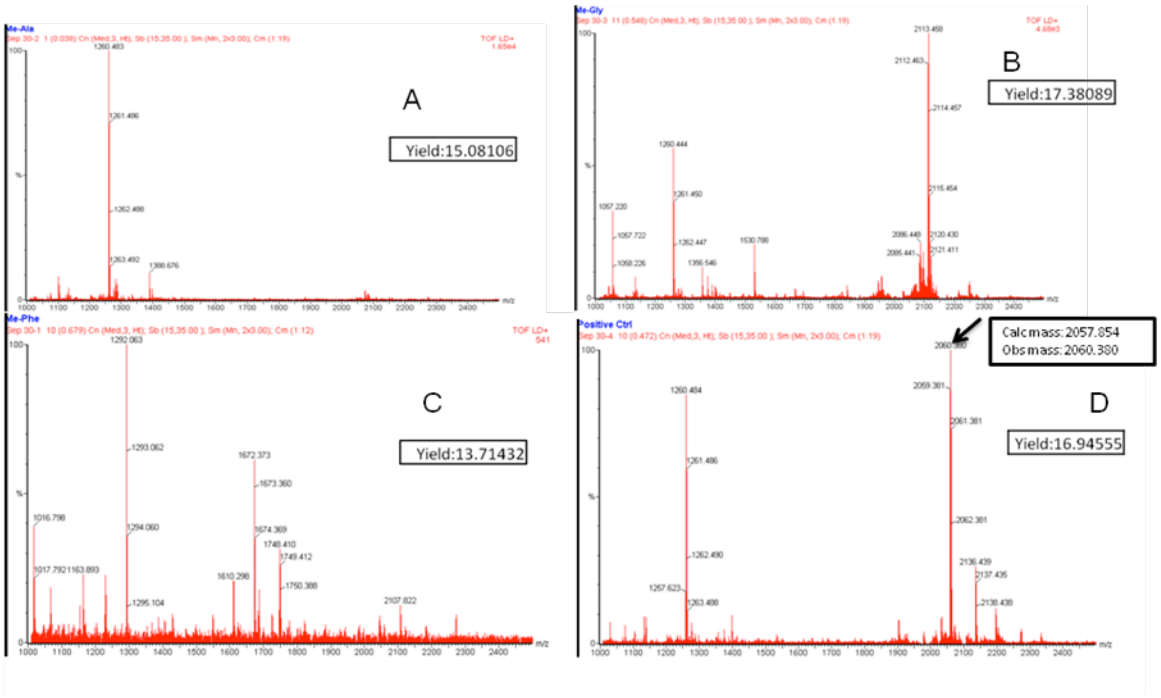


Figure 17. MALDI/MS analysis of 50 µL translations with A) incorporated alanine B) incorporated glycine C) incorporated phenylalanine and D) positive control

Yields were excellent for the 50 µL translation but desired mass peaks were not found on the MALDI/MS. Positive control which was a translation with natural amino acids had the peak corresponding to the correct as well as a 1260.48 peak. The 1260.48 peak corresponds to peptide consisting of Met-His-His-His-His-His-His-Met-Lys, which is part of the final peptide. This peak corresponds to translation termination before incorporation of Asn. For *N*-methyl alanine, *N*-methyl glycine and the positive control this 1260 peak was observed. In the case of *N*-methyl

phenylalanine, a peak at 1292 was detected, which most likely corresponds to the MHHHHHHMK peptide where the two methionine thioethers were oxidized.

In these experiments, the concentration of aminoacyl-tRNA added to the translation was 10 µM. This concentration was much less than 100 µM, the value was reported in literature. However, for a 50 µL translation this would require significant amounts of transcribed $tRNA^{Asn\text{-}E2}$. To solve the problem a smaller translation was designed. With 5 µl translation we could increase the concentration of aminoacyl-tRNA 10 fold. Therefore subsequent translations were done at a 5 µL scale.

Table 4 shows yields of single incorporation of three N-methyl amino acids in 5 µL translation. Except for the positive control no yield was obtained in the other assays. Also mass spectra from the three N-methyl amino acid assays did not show the expected masses.

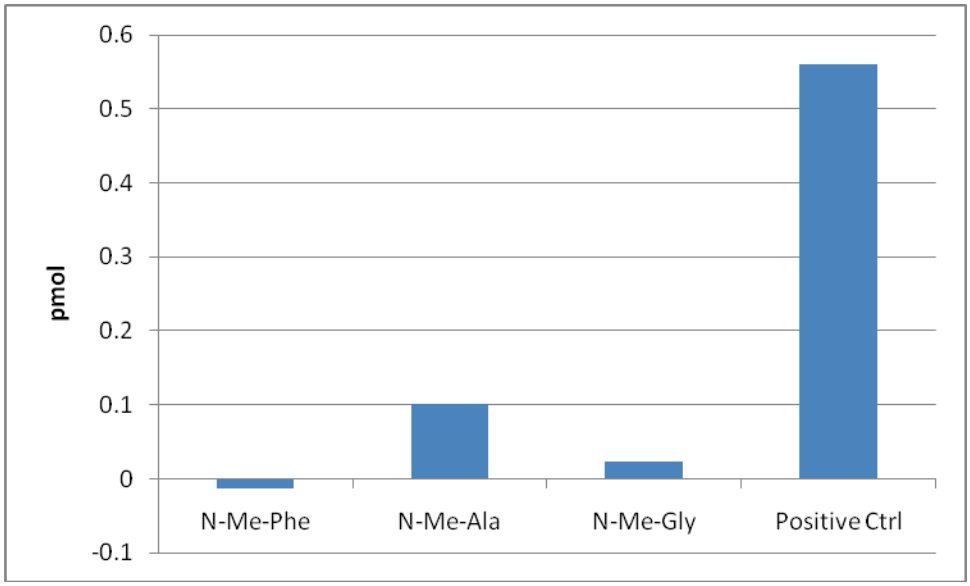


Figure 18. Translation yields for *N*-methyl amino acids (100 µM aminoacyl-tRNA)

Therefore the experiment was done with higher concentration (150 µM) of aminoacyl-tRNAs. Yields from this experiment are shown in figure 19. No peptides of the right mass were observed for the *N*-methyl amino acid experiments. This might be because of low yields of the peptides. A peptide of the correct mass was detected for the positive control, but it was much smaller than 1260 truncation peak.

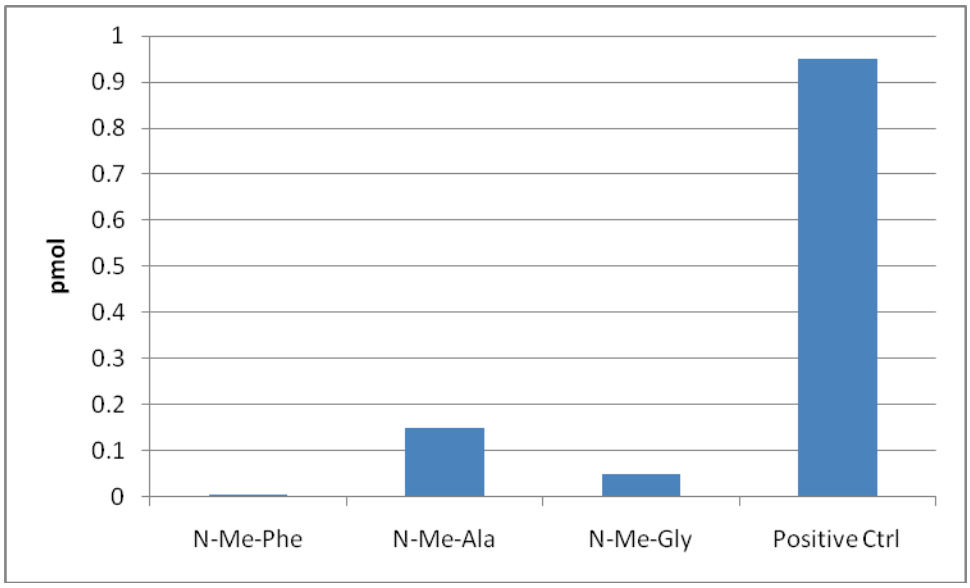


Figure 19. Translation yields for *N*-methyl amino acids (150 µM aminoacyl-tRNA)

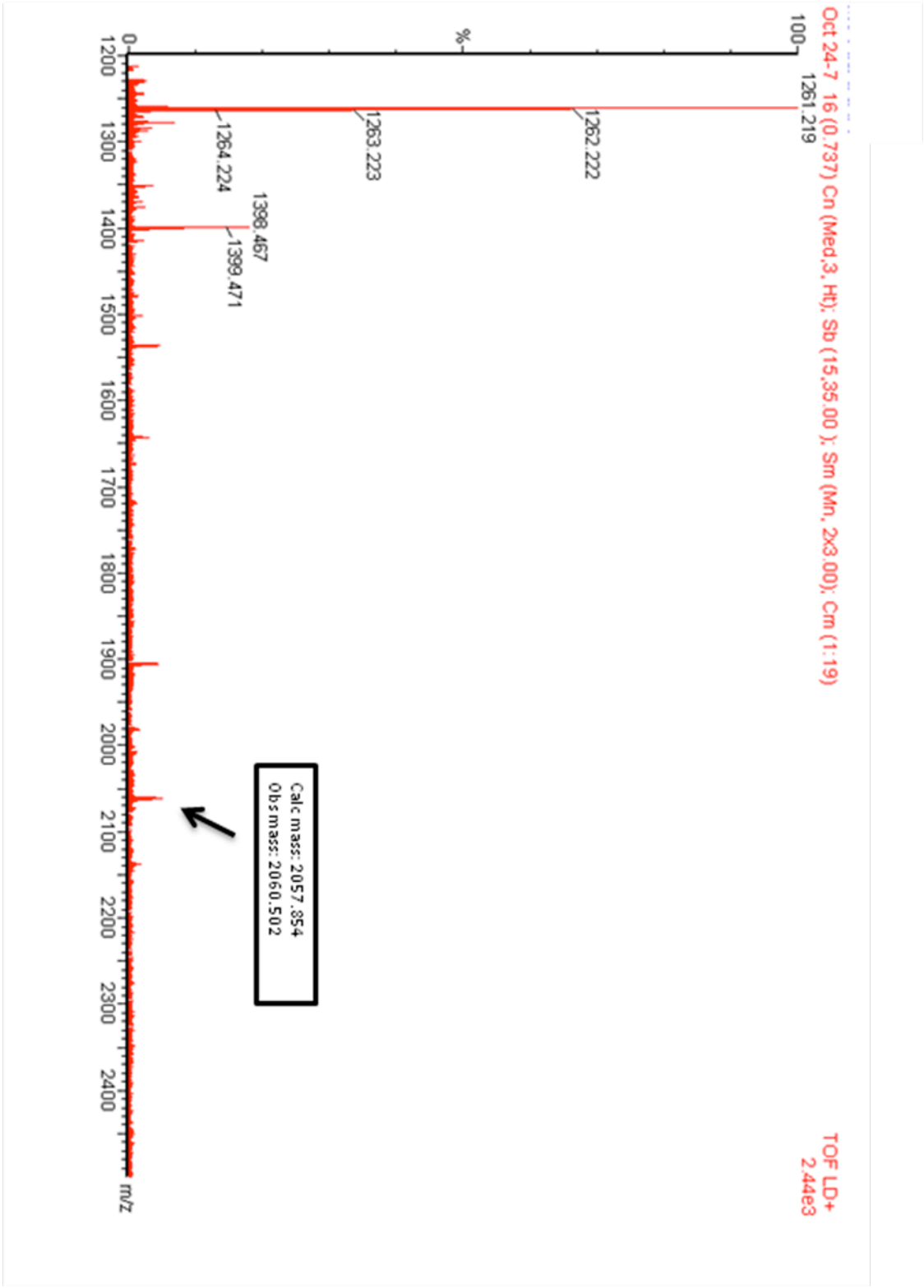


Figure 20. MALDI/MS analysis of incorporation of natural amino acids into the peptide

3.4 Short mRNA

In all above translations a peak at mass 1260, which is corresponding to $M(H)_6MK$, was observed (Figure 17 and 20). This might be because of inefficiency of the mRNA in complete translation. Therefore new mRNA was designed, which was shorter than previous one and encoded three amino acids. This mRNA should have less failure in its sequence and therefore more be more efficient. This short mRNA expresses a short peptide consisting of His tag peptide followed by Met-Phe-Asn-Cys. The efficiency of the mRNA was checked in a translation with natural amino acids and the yield of translated peptide was 3 pmol in 50 μL translation.

The new short mRNA was used in a translation with a Cys codon assigned to *N*-methyl glycine. No yield was obtained for this translation while positive control worked well, showing no truncation.

After these failures, we were concerned that our designer *N*-methyl AA/tRNA pairs were somehow incompatible with in vitro translation. We therefore decided to use the exact *N*-methyl amino acid and tRNA pairs that were reported by Suga and coworkers[2]. To this end, *N*-methyl serine was charged on $tRNA^{Asn\text{-}E2}$ (UUC). This pair was used in translation and the peptide with incorporated *N*-methyl serine was not detected while positive control gave a peptide of the correct mass.

Therefore we thought perhaps the experimental technique was needed to be improved. In all previous translations the aminoacyl-tRNA pellet, which was precipitated in ethanol was air dried for 5 minutes prior to dissolving in water for translation. We reasoned that the pellet might not have dried completely and residual ethanol may reduce translation yields.

The translation of *N*-methyl serine was repeated and this time the aminoacyl tRNA was dried carefully over argon. The right mass of peptide with incorporated *N*-methyl-serine was detected by MALDI/MS. The yield of the translation was 1.49 pmol in 5 µL translation.

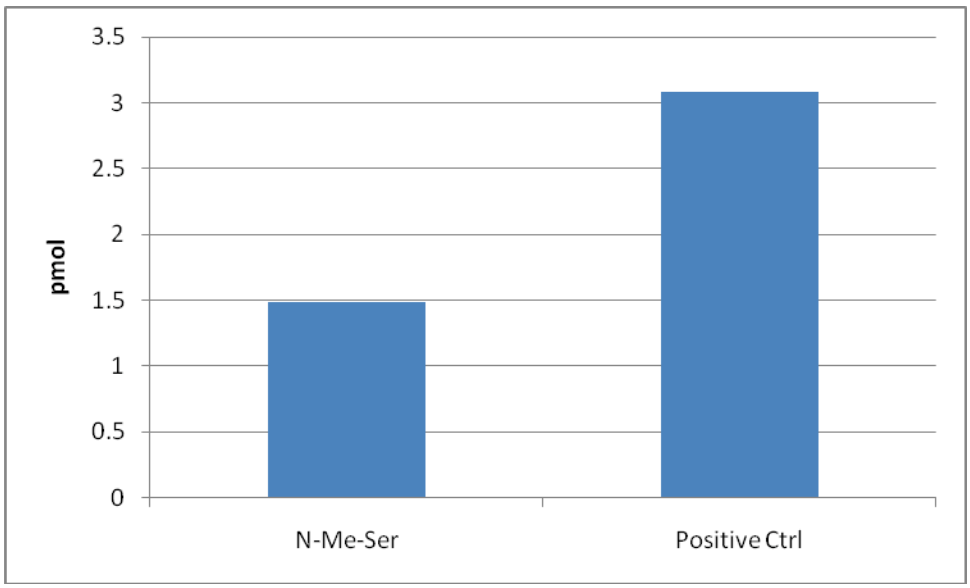


Figure 21. Translation yield of *N*-methyl serine

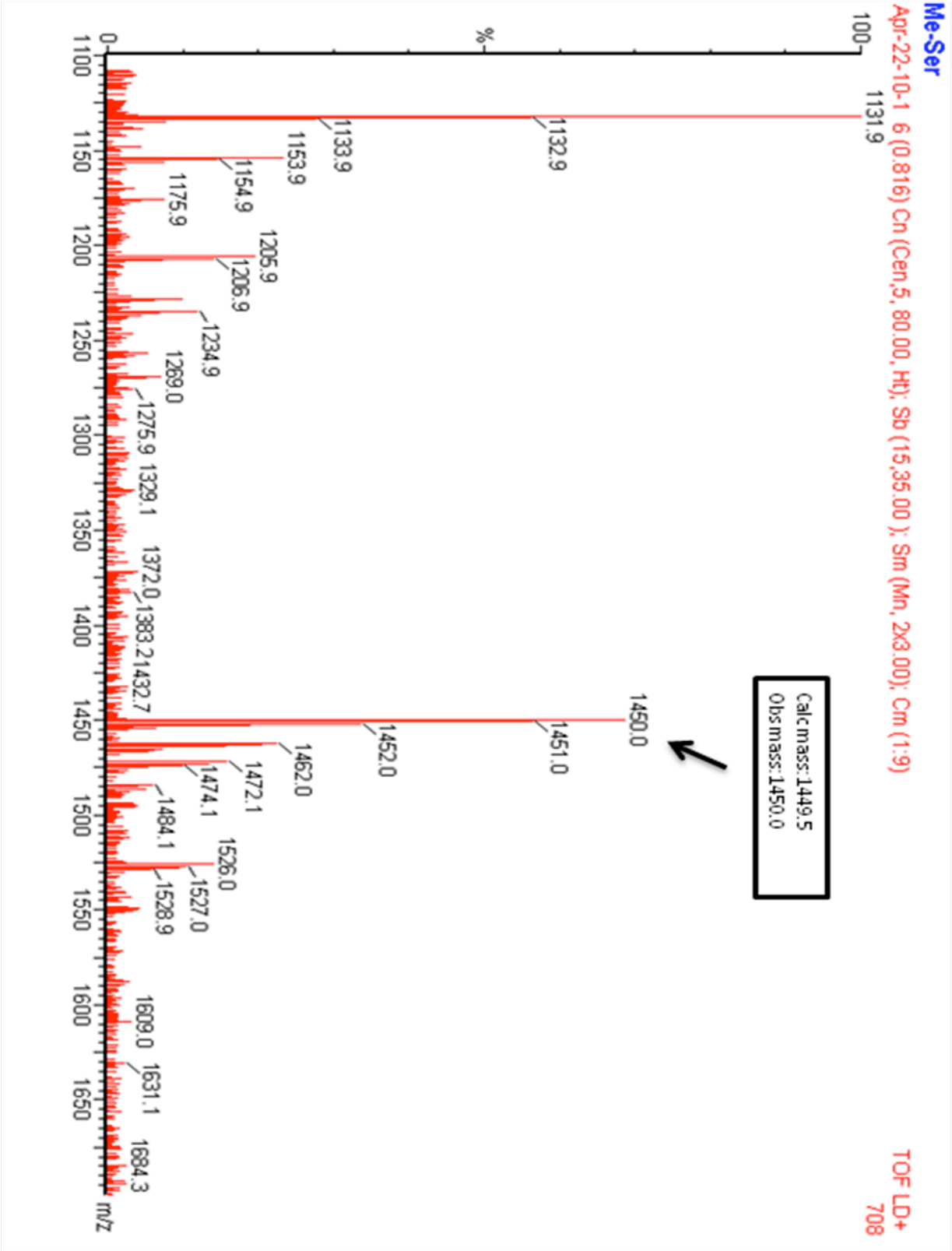


Figure 22. MALDI/MS analysis of incorporation of *N*-methyl serine into the peptide

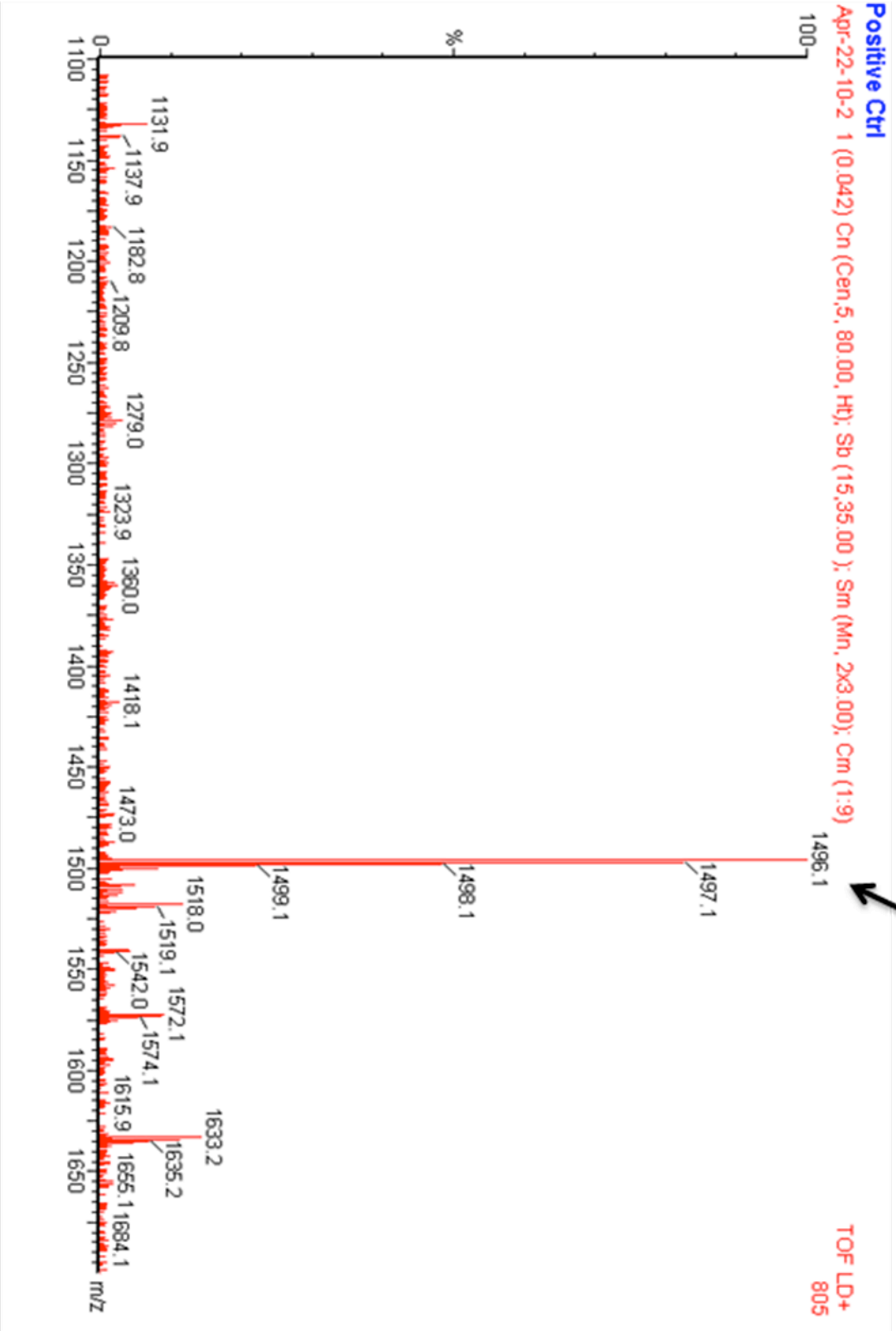


Figure 23. MALDI/MS analysis of incorporation of natural amino acids into the peptide

Using our new technique, *N*-methyl serine, *N*-methyl glycine and *N*-methyl phenylalanine were also tested in separate assays. Final peptide yields are shown in Figure 24. Peptide was produced in each assay, and MALDI confirmed the correct peptides were formed, except in the case of *N*-methyl phenylalanine. The mass was matched to the peptide with misincorporation of histidine instead of Asp codon. That could be because of low concentration of aminoacyl-tRNA (25 µM) that was used. In Me-Phe and Me- Ser spectra masses at 1131.8 and 1131.7 were observed respectively which is corresponds to MHHHHHHM truncated peptide. In case of *N*-methyl glycine, a peak at 1393 was observed that corresponds to MHHHHHHMFN and shows that translation was not efficient in incorporation of part of *N*-methyl glycine and terminated before Cys codon.

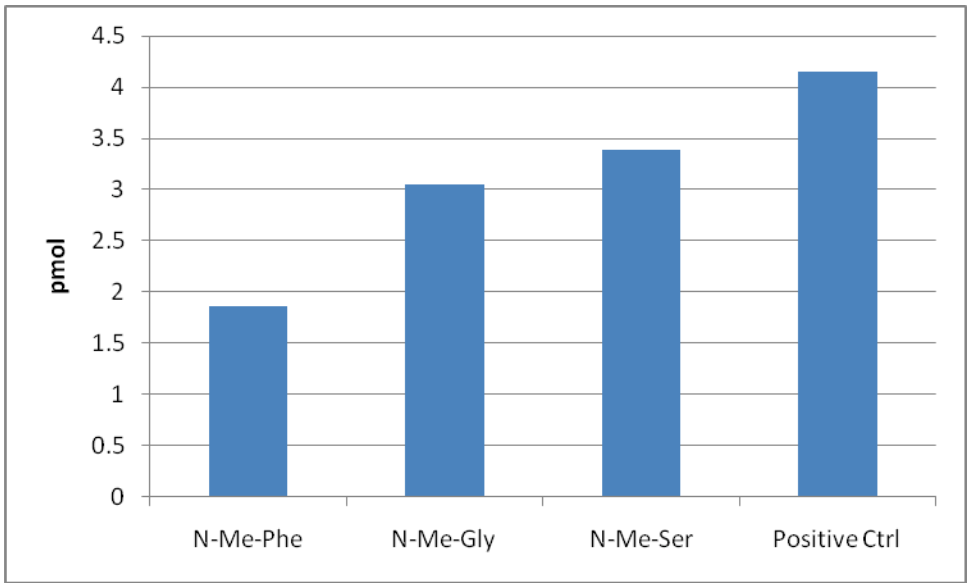


Figure 24. Translation yield of *N*-methyl serine, *N*-methyl glycine, *N*-methyl serine

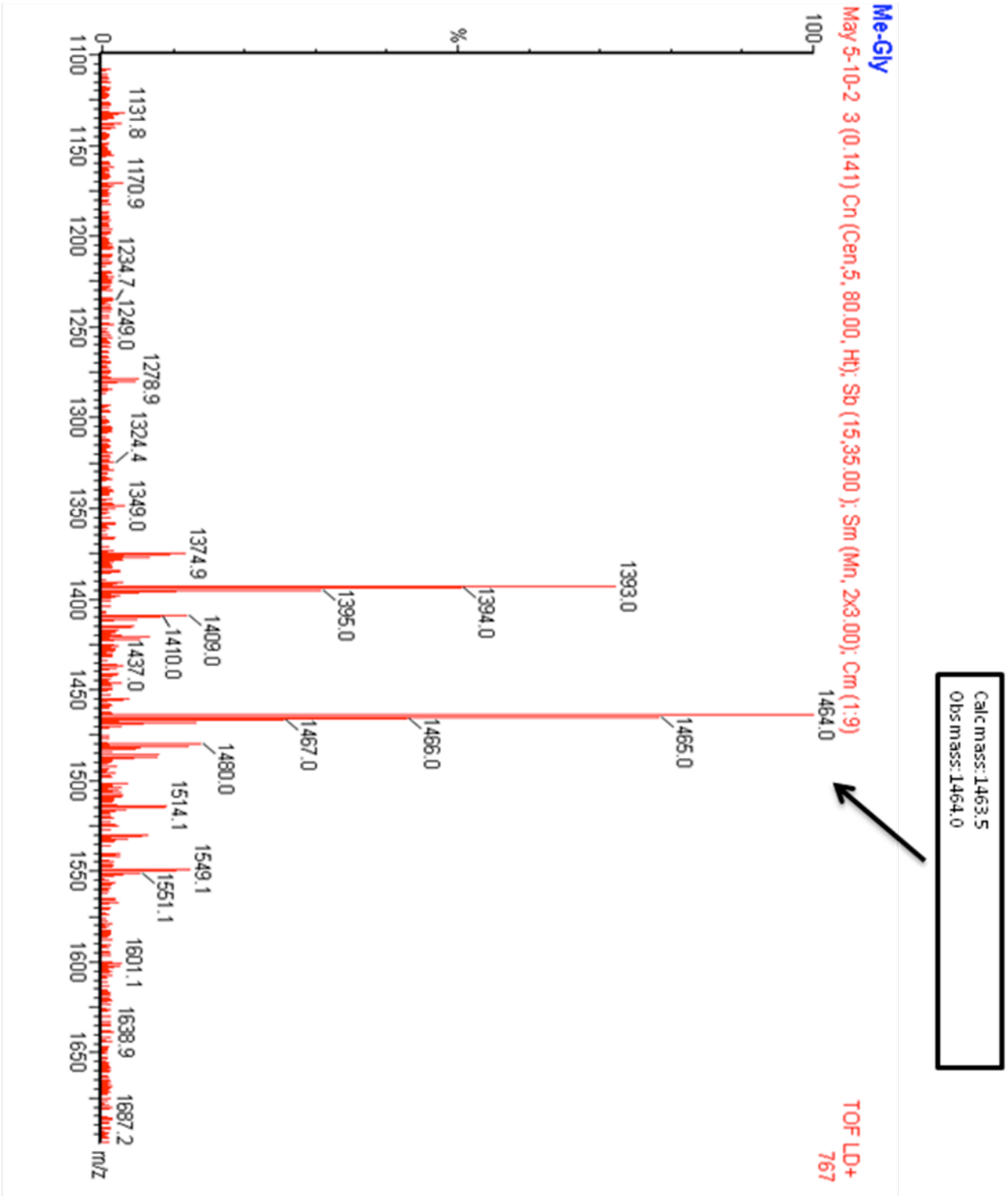


Figure 25. MALDI/MS analysis of incorporation of *N*-methyl glycine into the peptide

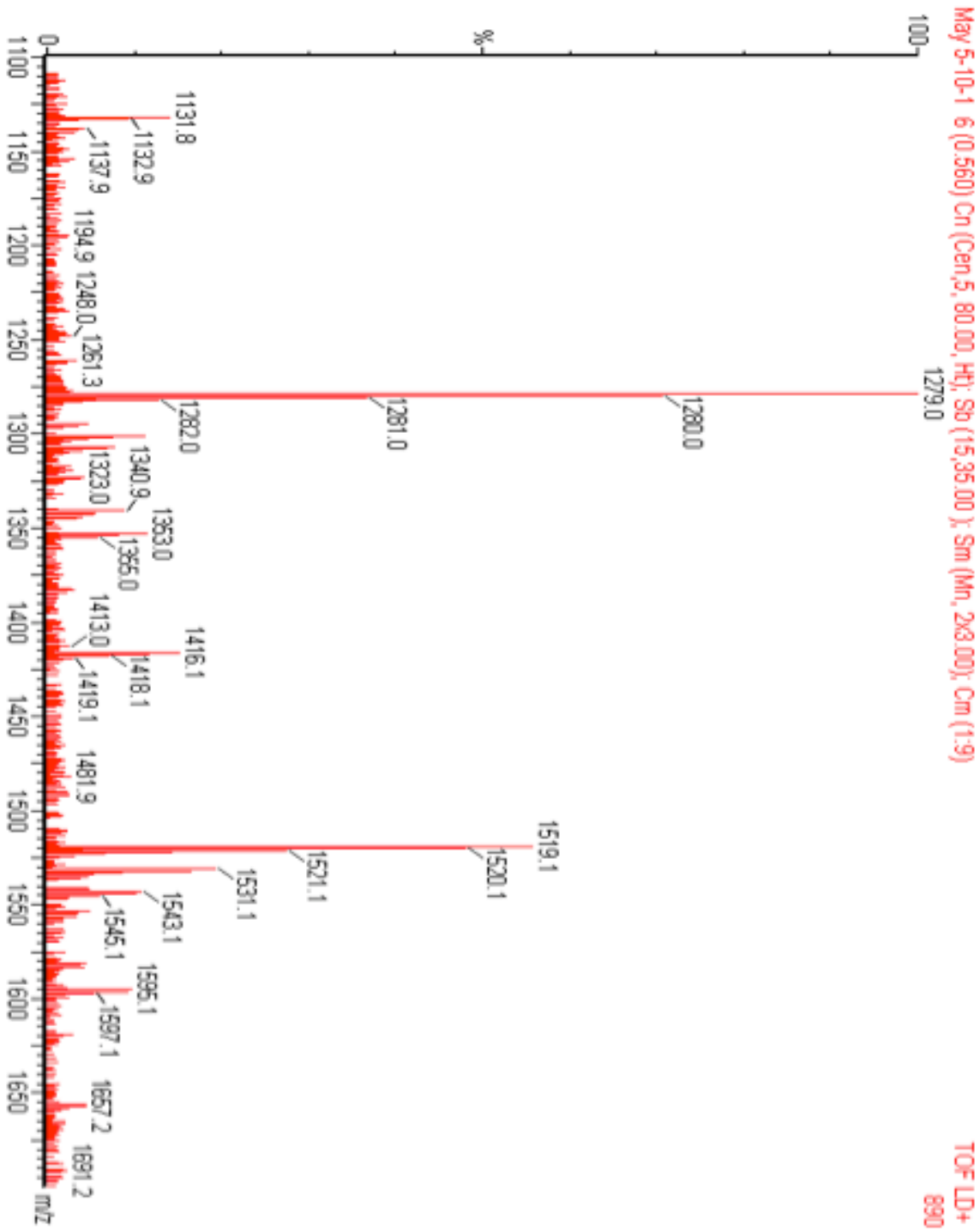


Figure 26. MALDI/MS analysis of incorporation of *N*-methyl phenylalanine into the peptide

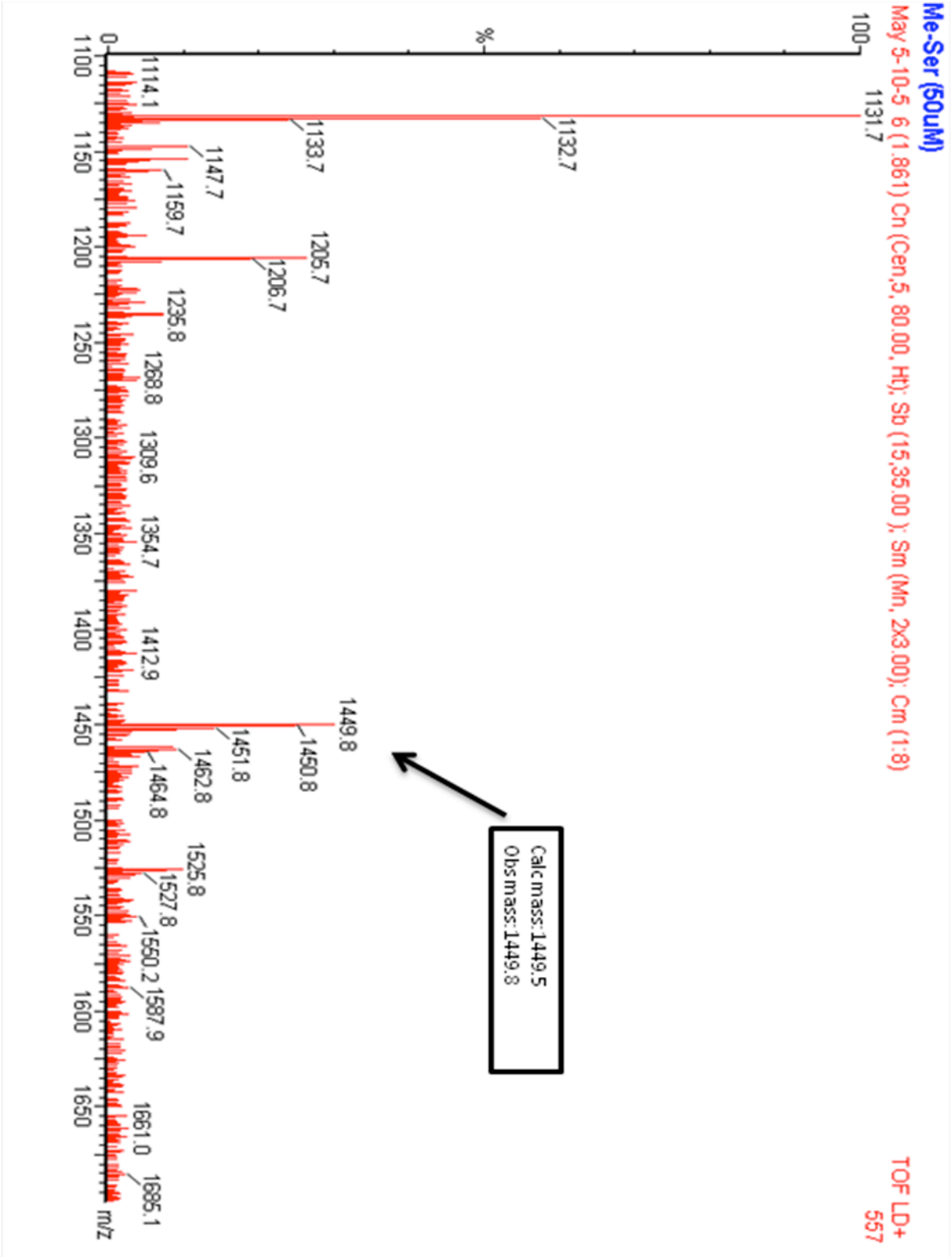


Figure 27. MALDI/MS analysis of incorporation of *N*-methyl serine into the peptide

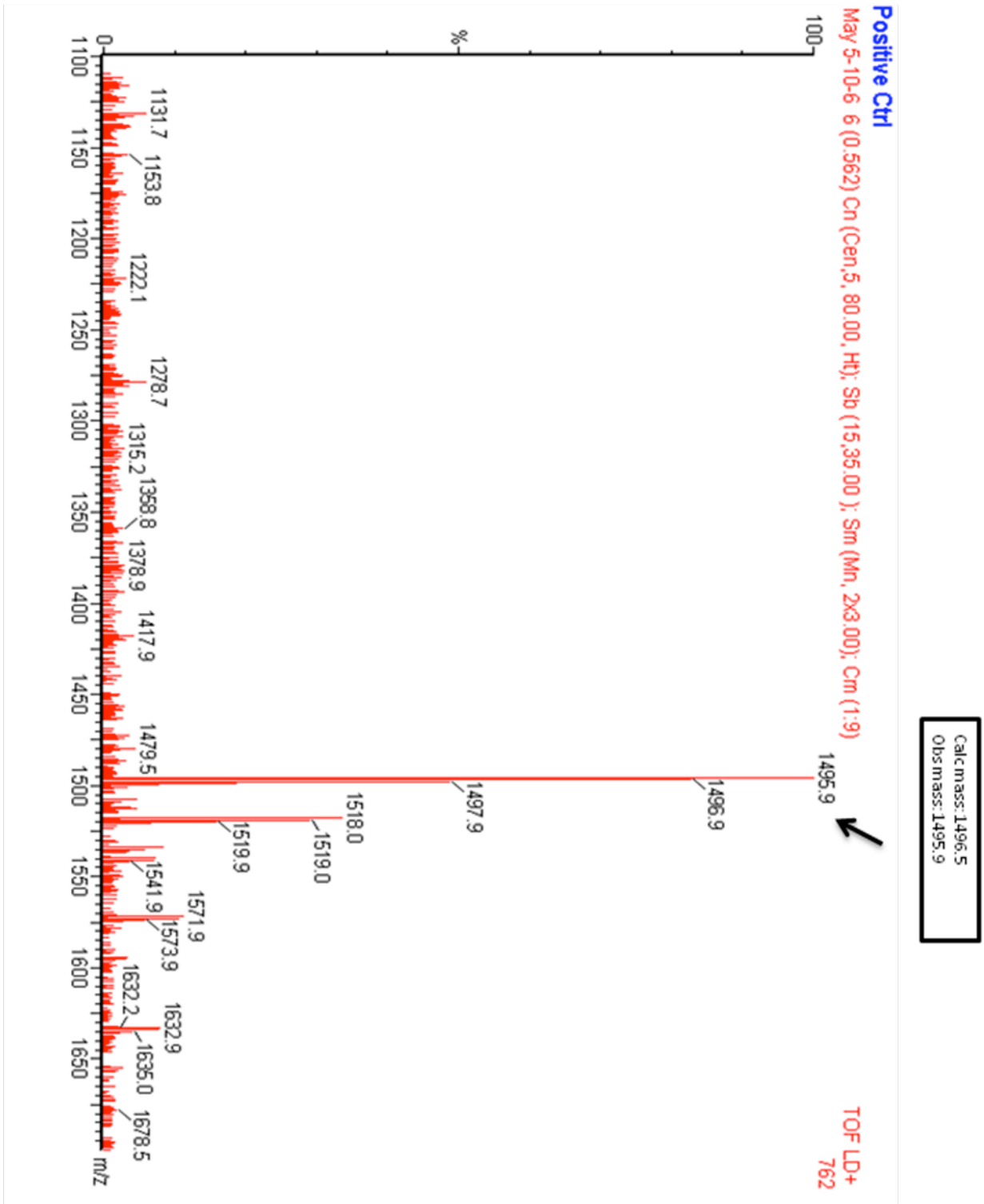


Figure 28. MALDI/MS analysis of incorporation of natural amino acids into the peptide

3.5 Experimental section

3.5.1 General methods

The DNA templates of mRNAs were synthesized on MerMade 4 DNA synthesizer.. Synthesized DNAs were deprotected with 1:1 solution of 40% methylamine and 30% ammonia mix solution and purified with 10% denaturing PAGE.

MALDI mass spectra were recorded using Micromass' M@LDI (MALDI TOF) micro channel plate spectrometer. 1 μL of sample was mixed with 9 μL of MALDI matrix (1% CHCA in 1:1 MeCN: 0.1% trifluoroacetic acid) and 1 μL was spotted on MALDI plate. Mass scans were obtained in the range of 1100-1700 m/z for short mRNAs translations and 1100-2500 m/z for longer mRNA.

Radioactivity of ^{35}S-peptides was counted with Beckman coulter LS 6500, multi-purpose scintillation counter.

3.5.2 PURE Translation

For the synthesis of N-methylated peptides, the PURE translation system was used. Translation reaction were carried out in presence of 1 μM mRNA and 0.1mM of methylated aminoacyl-tRNA and aminoacyl tRNA synthetase required for translation except the ones which were reassigned for *N*-methyl amino acids. In each translation 200 μM of natural amino acids were used except methionine, which 10μM concentration was used. In case of the positive control, PURE translation was used with 200 μM of the natural amino acids and all aminoacyl tRNA synthetase required. 10 μCi of ^{35}S methionine was used besides 20 μM of methionine in order to radiolabel the peptide. The translation mix was incubated for 3 hours in 37°C and quenched with

50 μL TBS (50 mM tris-HCl, [pH 8.0], 150 mM NaCl) buffer. The resulting peptide was captured on Ni-NTA resin by incubating for 1 hour at 25°C, washed with 500 μL TBS two times and was eluted with 50 μL 1% TFA solution. To measure the amount of translated peptide, peptides were labeled with ^{35}S methionine. 40 μL of sample was diluted with scintillation fluid (2 mL) and counted with a scintillation counter. Specific activity of the peptides was in range of 54183-173990 cpm/pmol.

3.5.3 MALDI-TOF-MS Analysis

The nonradiolable translated peptides were desalted with a C_{18} ziptip (Millipore) and eluted with 50% acetonitrile, 0.1% TFA solution saturated with matrix (α-cyano-4-hydroxycinnamic acid). 1 μL of sample was spotted on MALDI plate and detected with MALDI-TOF-MS.

3.6 Discussion

The goal of this part of the project was *in vitro* synthesis of *N*-methylated peptides. These experiments are complicated; however after optimization, the yields of the all-natural AA peptides reached 4.15 pmol in a 5 μL translation.

To efficiently incorporate *N*-methyl amino acids into the peptide, different concentrations of aminoacyl tRNAs were used. However even at high concentrations of aminoacyl tRNAs, no peptides containing *N*-methyl amino acids were observed. Because the translation reactions with all natural AAs worked efficiently, we concluded that there might be something wrong in our preparations of the *N*-methyl aminoacyl-tRNAs we introduced into the translation system. Therefore the aminoacyl tRNAs were dried very carefully under argon and dissolved in fresh 1mM NaOAc pH 5.0. *N*-methylated peptides were obtained after this technical improvement.

Among the *N*- methyl amino acids that were used, *N*-methyl phenylalanine was not incorporated to the peptide efficiently. This might be because of the low concentration of the aminoacyl tRNA (25 μM) that was used.

The next step is making a library of *N*-methylated peptides and for screening with a cell permeability assay. But to make a library of peptides, translation reactions of up to 5 ml are needed. In such a large-scale translation, a large amount of transcribed tRNA is also needed.. Therefore we decided to use tRNA from another source instead of transcription from DNA template. The next chapter focuses on purification of specific tRNAs from total tRNA.

Chapter 4

Purification of individual tRNAs from total tRNA

New study was done on purification of individual tRNAs from total tRNA. By tagging specific tRNAs with fluorous compound, they can be purified with fluorous-pak column. After deacylation, purified tRNA can be eluted from column and be used in translation.

4.1 Purification of specific tRNAs from total tRNA

As outlined in chapter 3, it is necessary for our experiments to obtain large amounts of individual tRNAs. Individual tRNAs can be made by *in vitro* transcription but lack of modifications of the tRNA transcripts may influence their function[23, 24]. Therefore developing a simple and rapid method for purification of specific tRNA from fully modified E. coli total tRNA would be advantageous.

The purification of specific tRNAs from total tRNA is a process that requires multiple chromatographic steps[25]. People have tried to purify specific tRNAs by hydrophobic tagging by synthesizing Fmoc-aa-tRNA species. This technique has the advantages over previous methods[26]. For example, it is applicable for all tRNAs because it relies only on the selective aminoacylation it is cheap and the ester used in this study (FmocOSu) is stable and available at low cost. But this technique has been applied only to two types of tRNAs and separations require HPLC.

4.1.1 Using fluorous affinity tag in purification of specific tRNAs

Fluorous–highly fluorinated-compounds have been used in different targeted synthesis, purification of small molecules, peptides and oligonucleotides[27]. Seperation of compounds base on fluorous tag is really reliable. The utility of fluorous-based methodologies has been extended recently.

We decided to try to extend fluorous tagging and purification to isolate individual tRNAs. In order to this, desired tRNA will be charged with related amino acid. Using reductive amination reaction, the charged tRNA will be coupled with fluorous molecule. This tagged tRNA can stick to fluorous-pak column while others will pass through and be removed. To release the tRNA from column, tRNA can be deacylated and then eluted with acetonitrile.

4.2 Purification based on fluorous affinity

The commercially available fluorous molecule 4-(4,4,5,5,6,6,7,7,8,8,9,9,10,10,11,11,11-Heptadecafluoroundecyloxy) benzaldehyde (1) (Figure 16) was chosen for this study. We reasoned that the aldehyde group could react via reductive amination to attach the fluorous tag to aminoacyl-tRNA, a reaction similar to our MALDI AARS assay.

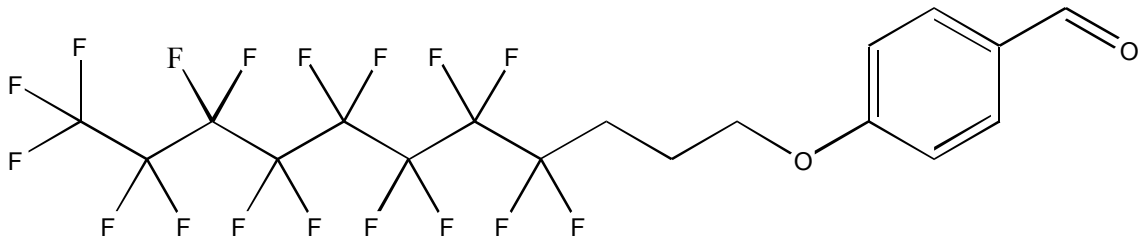


Figure 29. Fluorous molecule (1)

4.2.1 Solubility

The biggest problem with this fluorous compound is that it iis not water-soluble and therefore not compatible with our tRNA study. Many experiments were tried using organic co-solvents for the fluorous molecule but none were successful. Therefore we decided to sulfonate our fluorous molecule to make it more water-soluble.

4.3 Sulfonation of compound (1)

In order to sulfonate the fluorous molecule, first sulfuric acid was tried. The first reaction used 30% sulfuric acid. But the fluorous molecule was not soluble. The reaction was run for 24 hours and followed by TLC and NMR. No product formed.

4.3.1 Solvent

The solubility of the fluorous compound in various water miscible solvents was tested. The results are shown in table 5. However no solvents that were both able to dissolve the compound and unreactive to sulfuric acid were found.

Table 5. Solubility of compound (1) in different solvents

Solvent	Dissolved the sample	Miscible with water
MeOH	No	Yes

DCM	No	No
DMF	Yes	Yes
Ethyl acetate	No	Yes
Acetone	Yes	Yes
DMSO	Yes	Yes

4.3.2 Sulfuric acid percentage

Therefore we have decided to do the reaction without any co-solvent but by increasing the concentration of sulfuric acid and therefore decreasing the water percentage. Experiments were designed in which different percentages of sulfuric acid were used. Table 6 shows the result of experiments with different concentrations of sulfuric acid. As expected the solubility was inversely proportional to the concentration of water. Reactions were followed by TLC and NMR. In all cases the molecule remained as starting material.

Table 6. Different sulfuric acid percentages

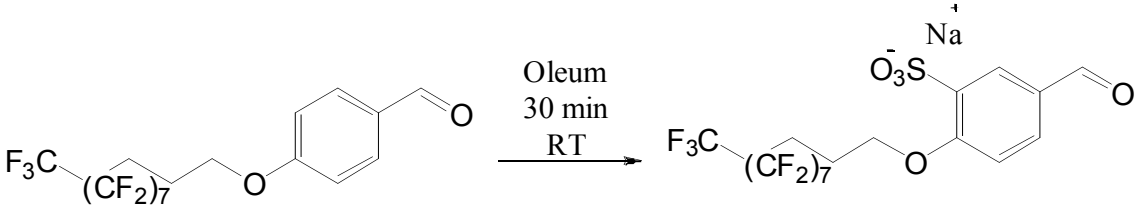
Sulfuric acid conc.	Solubility of starting material	Product

Sulfuric acid 20%	Not soluble	No
Sulfuric acid 40%	Partially soluble	No
Sulfuric acid 60%	Partially soluble	No
Sulfuric acid 80%	Partially soluble	No
Sulfuric acid 98%	Soluble	No

4.3.3 Temperature

After trying different sulfuric acid percentages, we decided to heat to push the reaction. Different temperatures were tried: 50°C, 75°C and 125°C. In case of 125°C color of reaction was changed. In all cases, reaction was followed by TLC, which did not show any progress in reaction. ^{1}HNMR spectrum of the sample in 125°C showed a messy spectrum which did not match desired product.

Finally we decided to use oleum, which is fuming sulfuric acid instead of normal sulfuric acid for this reaction. Oleum 25% was used and sample was completely soluble. The product was formed after 30 min. ^{1}HNMR confirmed the formation of the product. The yield of the reaction was 72%. The final product did not need to be purified, and our delight was completely water-soluble.



Scheme 4. Sulfonation of compound (1)

4.4 Fluorous column

Fluorous column was used in order to purify the individual tRNAs. Figure 30 shows the procedure followed in purification of fluorous-tagged tRNAs.

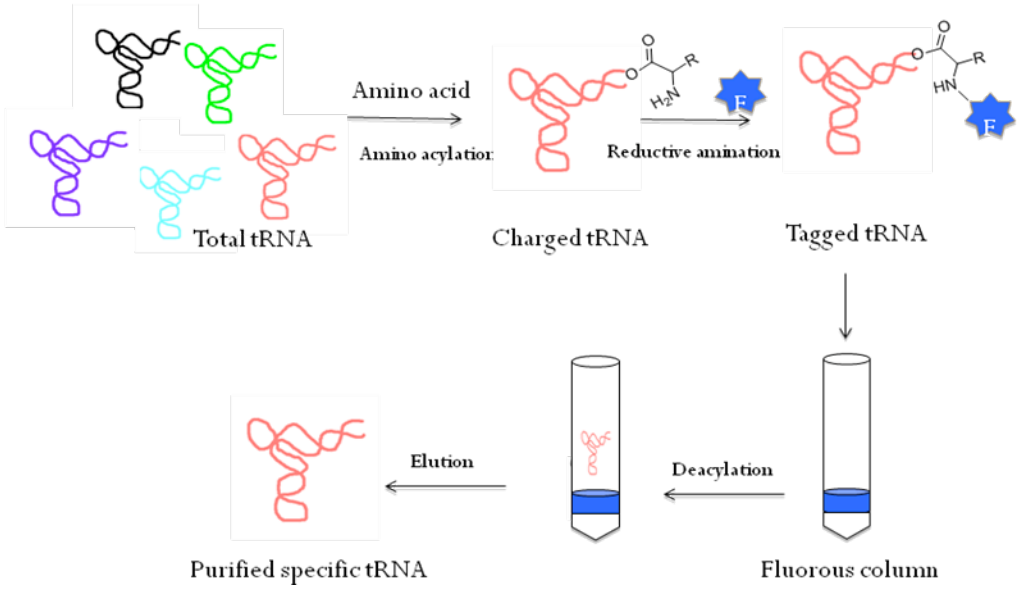


Figure 30. Preparation of fluorous tagged tRNA and purification on column (F= Fluorous molecule)

4.5 Labeling tRNA with ^{32}P

To efficiently follow the aminoacyl-tRNA samples on column, we have decided to label the tRNA with ^{32}P-ATP and follow the purification on column with radioactivity. The ^{32}P labeling method was described in section 2.6.

4.6 Loading cahrged tRNA on column

Radio-labeled total tRNA was first charged with all natural amino acids and synthetases. The tRNA was reacted with the sulfonated fluorous compound and then passed through the fluorous-pak column. Two different techniques were tried for deacylation of the tRNA on column: tris buffer pH 8.8 and 0.01 M cupper solution. At the end sample was eluted with 20% ACN.Figure 31 shows the experiment results.

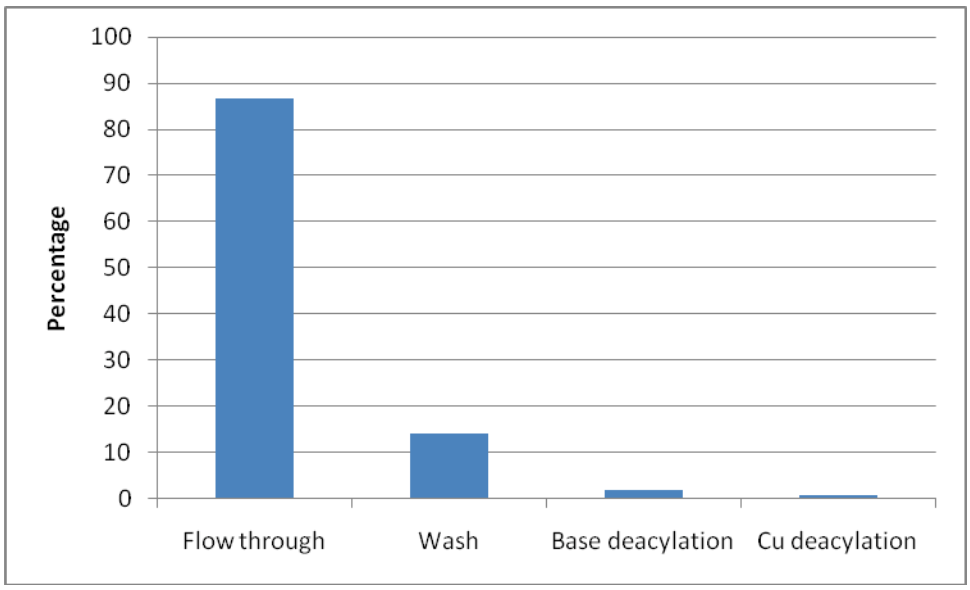


Figure 31. Purification of charged tRNA on fluorous column (Y axis shows the percentage of total input radioactivity)

The data shows that very little tRNA bound to the column. This might be because the amino acid charging assay did not work or failure in reductive amination reaction. Chraged tRNA was tested with ^{32}P PEI TLC technique and result showed a failure in labeling tRNA with ^{32}P.

Therefore we repeated the ^{32}P labeling tRNA with modifications such as increasing the total tRNA concentration and decreasing the ATP concentration.

The labeled tRNA was charged with all 20 natural amino acids and tagged with the fluorous compound through a reductive amination reaction. The final compound was loaded onto the fluorous affinity column. Figure 32 shows the result of this experiment. ^{32}P counting showed that most of the sample passed through the column in the loading/washing steps. However 11% was eluted after treating with tris and elution with 20% ACN. But less than 2% was eluted with cupper. Because the column was still radioactive, we tried to expose the column to tris in longer time (1 hour) and elute it with 20% ACN.

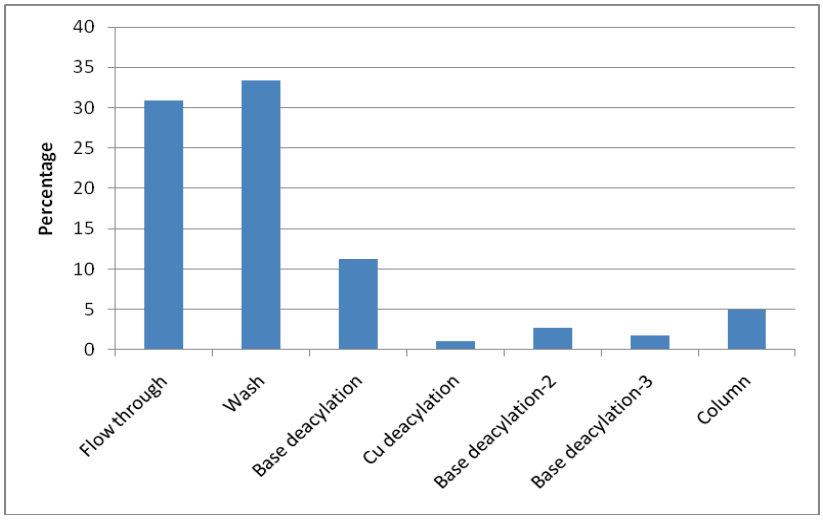


Figure 32. Purification of charged tRNA on fluorous column (Y axis shows the percentage of total input radioactivity)

Since we would expect that only tRNAs charged with amino acids would be fluorous tagged, we decided to determine the percentage of the total tRNAs that were AA-tRNAs in order to get an idea of the upper limit of what we could expect to be bound to the fluorous column. This was done using PEI-TLC method (Section 2.6). The phosphorimage showed that 52% of the tRNA was charged and 3% of whole radioactive sample was ATP. This modification applied to the calculation and showed us that approximately 23% of charged tRNA was eluted from column by treating column with ACN.

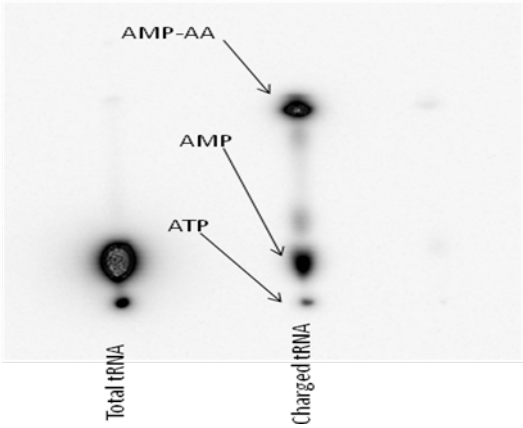


Figure 33. PEI TLC of charged tRNA

4.7 Experimental section

4.7.1 General experimental methods

4-(4,4,5,5,6,6,7,7,8,8,9,9,10,10,11,11,11-heptadecafluoroundecyloxy) benzaldehyde and oleum 25% were purchased from sigma. Fluorous-pak columns were purchased from Berry and

Associates. Images of PEI TLC plates (Sigma) were taken from Typhoon 9410 phosphoimager from GE Healthcare Company.

4.7.2 Synthesis of sodium 4-(4,4,5,5,6,6,7,7,8,8,10,10,11,11,11-heptadecafluoroundecyloxy sulfonate

100 mg of aldehyde (1) was added to 160 µL of fuming sulfuric acid (oleum) and stirred for 30 min in room temperature. The product was added to 3 mL of brine and the precipitate was filtered and dried under vacuum. 72 mg white solid was left (72%). ^{1}H NMR ($CDCl_3$, 400MHz) δ 9.86 (s, 1H), 8.21 (d, 1H), 7.78 (s, 1H), 7.17 (d, 1H), 4.25 (t, 2H), 1.76 (m, 3H), 1.97 (m, 2H).

4.7.3 Charging assay

Amino acids were charged onto total tRNA by aminoacyl-tRNA synthetases. 200 µM of amino acids and 350 µM of total tRNA were used in presence of BSA (10 mg/ml) and 2-mercaptoethanol (100 mM). HEPES buffer pH 7.4 (300 mM), 1M KCl and 1M $MgCl_2$ were added and the reaction was allowed to proceed at 37°C for 30 min. The reaction was quenched with 3M NaOAc pH 5.2 (0.1 Vol) and aminoacyl tRNA was recovered from phenol: chloroform: isoamyl alcohol 23:24:1 extraction and ethanol precipitated.

4.7.4 Reductive amination

The pellet from charging assay was dissolved in 12.5 µL 200mM NaOAc pH 5.0 and half of that was used in reductive amination. 12.5 µL of 69 mM sulfonated fluorous tag was added with 2.5 µL of 200mM $NaCNBH_3$.The reaction was allowed to proceed at 37°C for 2 hours and was quenched with 0.1 volume of 4.4 µL of NH_4OAc and 3 volume of 100% ethanol. The tRNA was allowed to precipitate and the resulting pellet was washed two times with EtOH 70% and two

times with EtOH 100%. The pellet was allowed to dry and then dissolved in 1 mL sodium acetate pH 5.2.

4.7.5 Fluorous column

Fluorous-pak column (Berry & associates) was preconditioned with first 2 mL ACN, then 2 mL 0.1M TEAA, and finally 2 mL of water with loading rate of 2 seconds per drop. After that, 2 mL of sample diluted 1:1 with loading buffer (5% DMF, 10% NaCl in water) was loaded on the column with 5 seconds per drop loading rate[28]. The column was washed with 2 mL of 10% ACN in 0.1M of TEAA solution pH 7 and 2 mL of water. In the next step tris pH 8.8 (1 hr)[29] or 0.01M copper was used for deacylation and the column was washed again with 1 mL 0.1M TEAA and 1mL of water. Finally the sample was eluted with 20% ACN, 50% ACN or MeOH. A portion of the sample was analyzed using a scintillating counter.

4.8 Discussion

A fluorous tag, which was sulfonated and therefore became water-soluble, was coupled with individual tRNA in total tRNA solution and purified with fluorous-pak column. The desired tRNA was deacylated on the column with tris buffer pH 8.8 and eluted with 20% ACN. Approximately 23% of the charged tRNA was recovered from the column. This initial result is promising and there are several parameters that could possibly be optimized. For example, the time of reductive amination reaction or deacylation could be varied to maximize yields or recovery.

Conclusions

Flexizyme system and PURE translation was used to synthesis *N*-methylated peptides. After optimization of translation peptides with incorporated *N*-methyl amino acids were obtained. However truncated peptides and misincorporation were observed in MALDI/MS spectra of translated peptides. Low concentrations of aminoacyl-tRNAs could be the reason of translation terminations.

Using Flexizyme for preparation of aminoacyl tRNAs requires a significant amount of transcribed tRNA in order to make library of N-methylated peptides; therefore, anew project on purification of specific tRNAs from total tRNA was started.

Desired *E. coli* tRNA was tagged with fluorous molecule and purified with fluorous-pak column. This tRNA can be charged with any amino acid by Flexizyme and be used in PURE translation. Libraries of *N*-methyl amino acids can be made and screened for peptides with suitable drug properties.

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Appendix

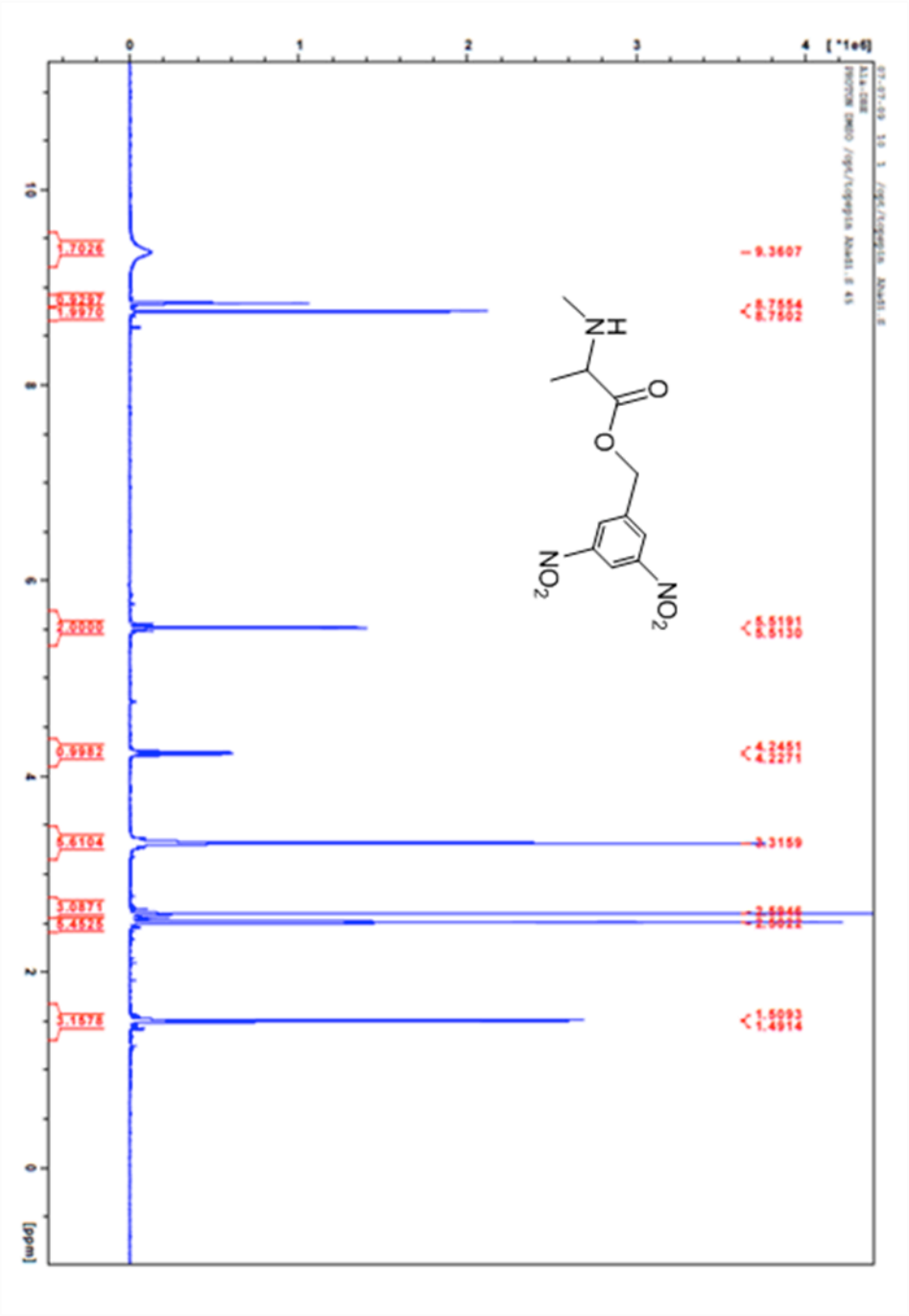

Figure 34. ^{1}H NMR of dinitrobenzyl ester of *N*-methyl alanine

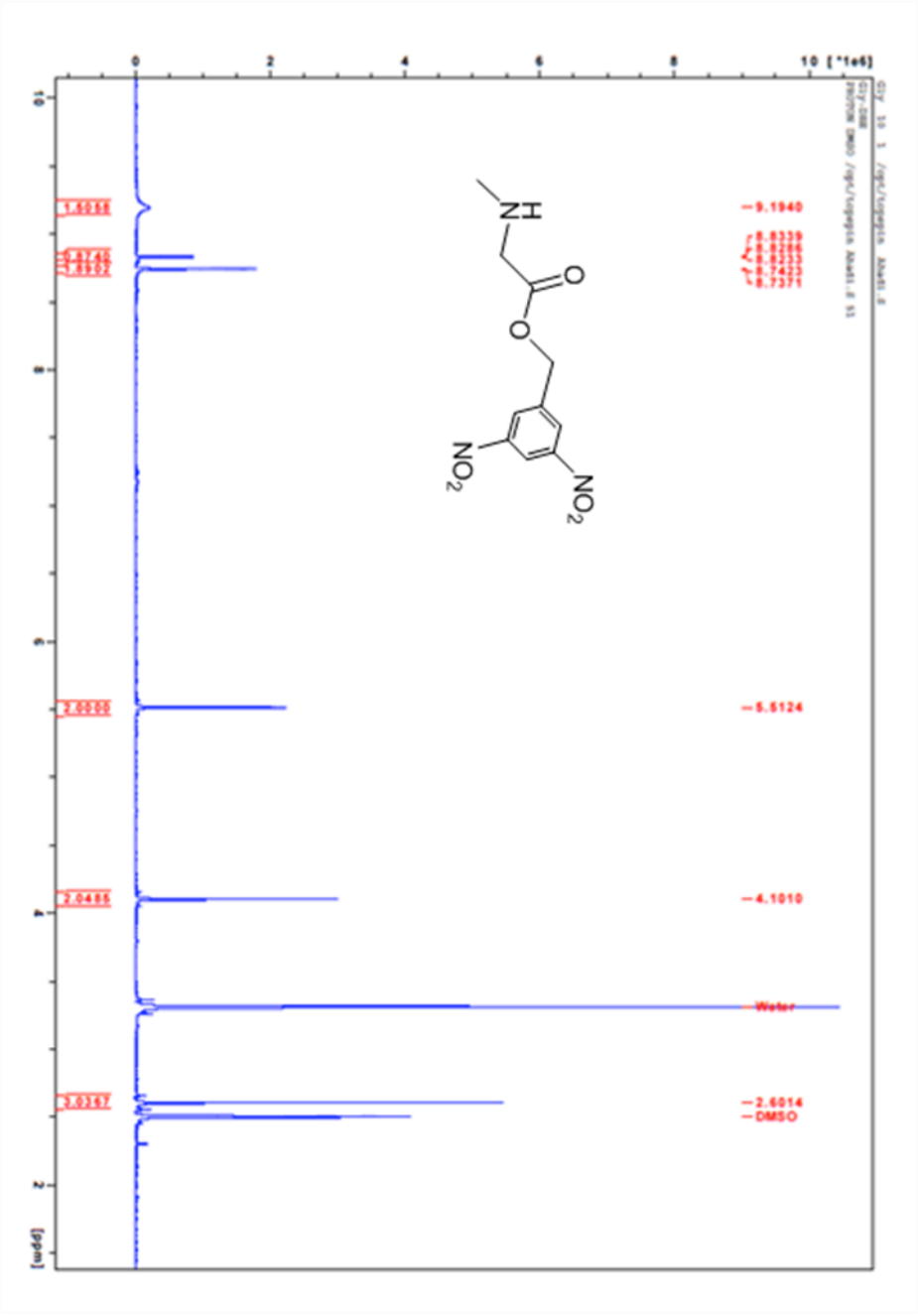

Figure 35. ^{1}H NMR of dinitrobenzyl ester of *N*-methyl glycine

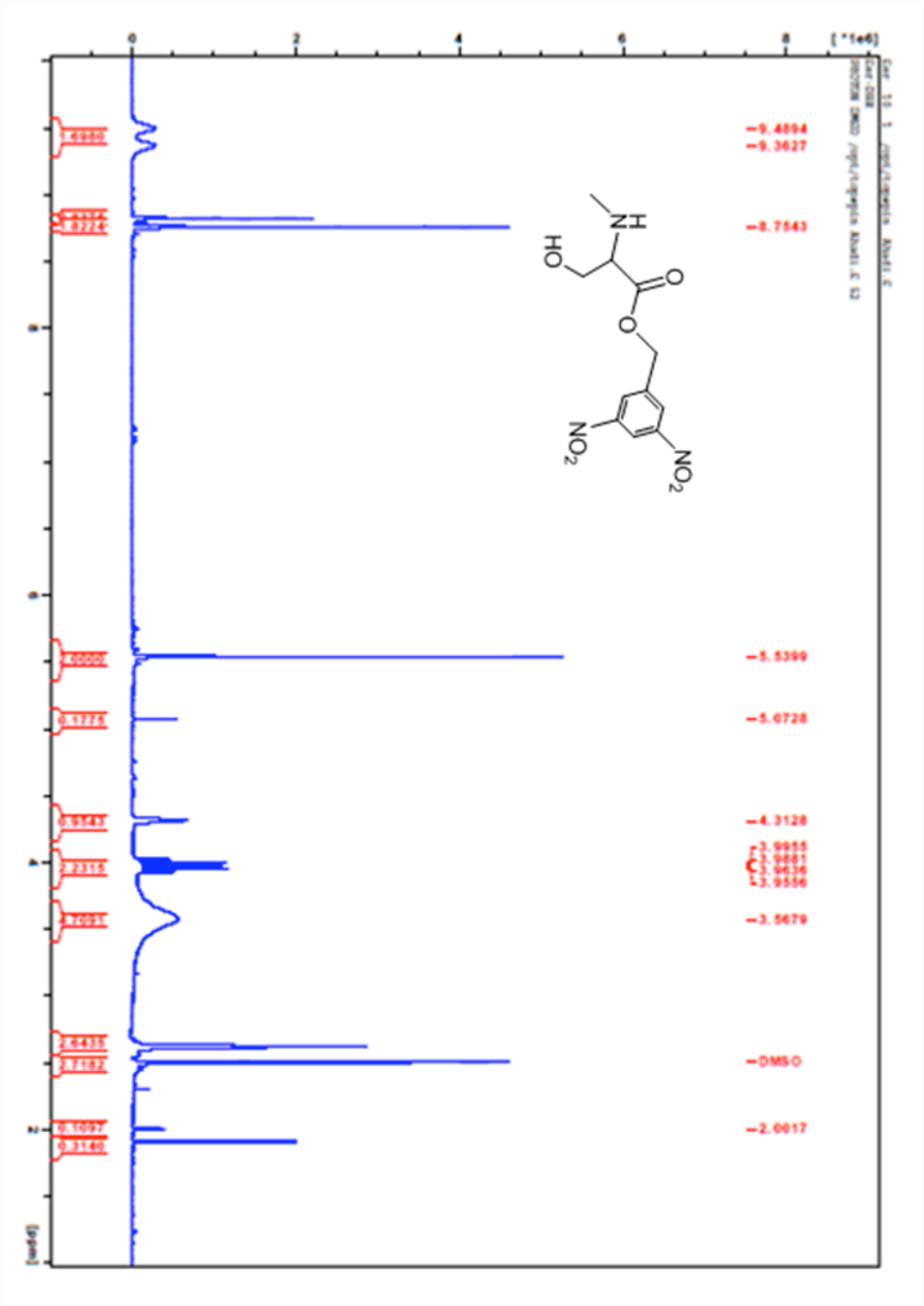

Figure 36. ^{1}H NMR of dinitrobenzyl ester of *N*-methyl serine

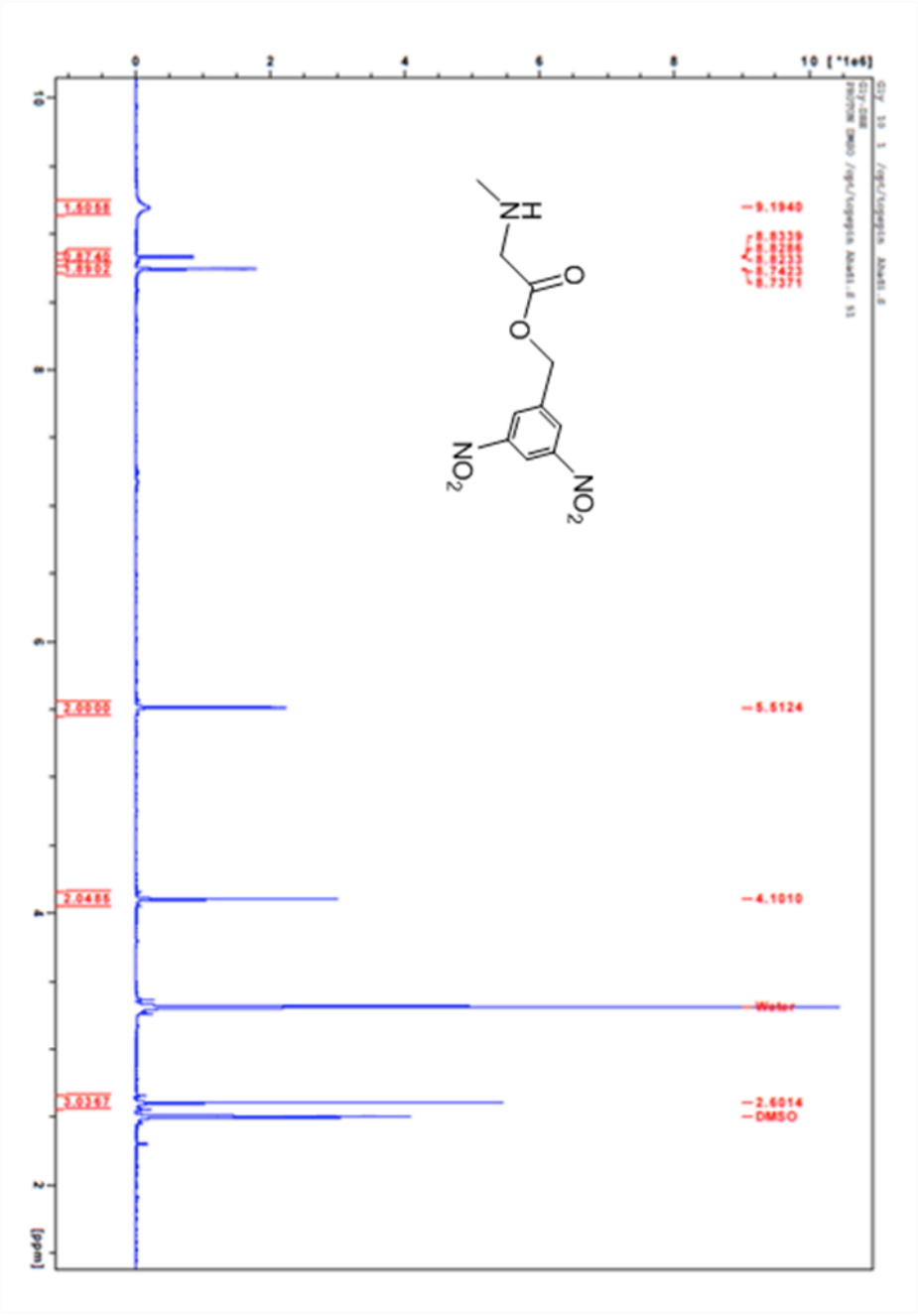

Figure 37. ^{1}H NMR of cyanomethyl ester of *N*-methyl phenyl alanine

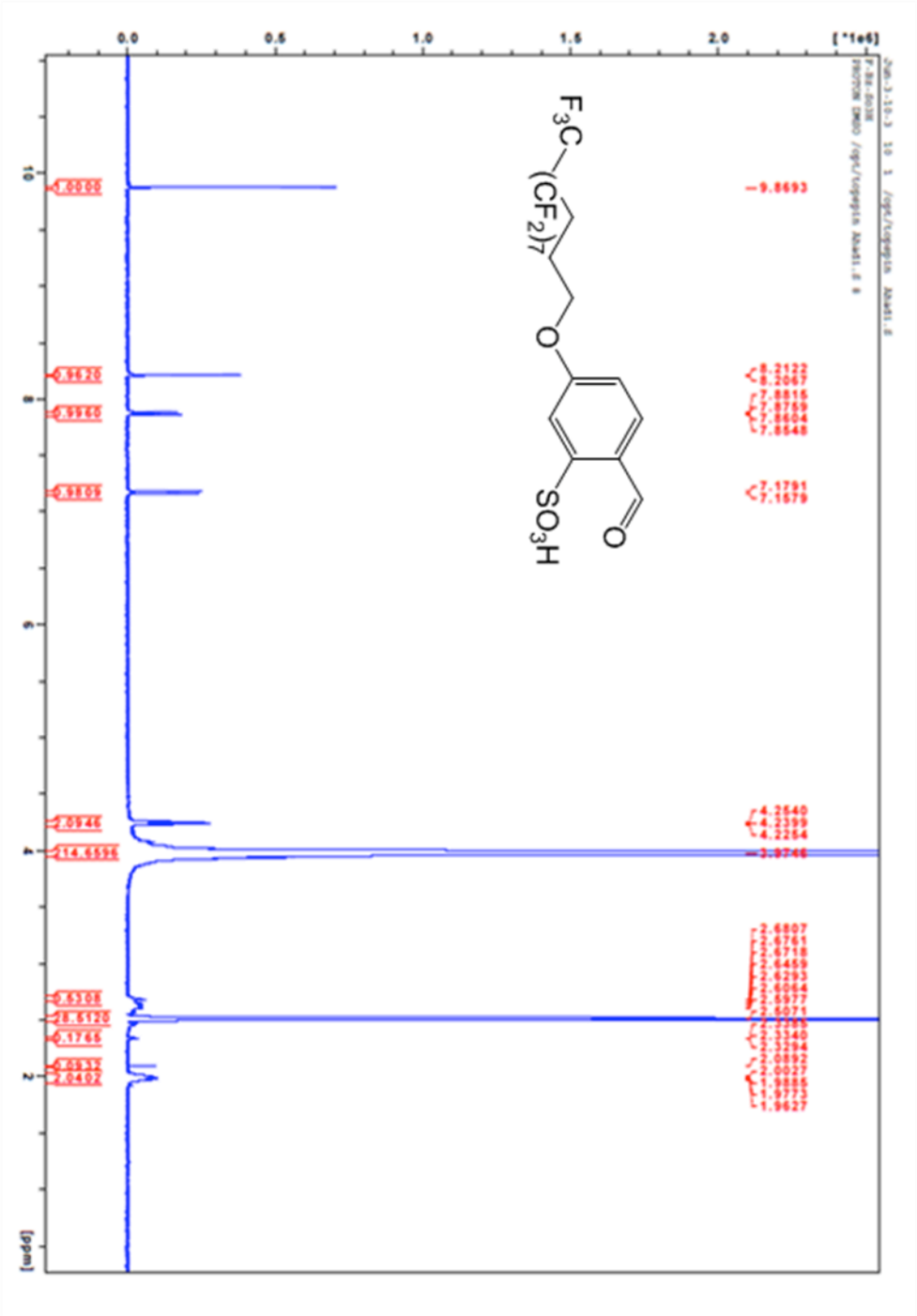

Figure 38. ^{1}H NMR of sulfoanted salt of 4-(4,4,5,5,6,6,7,7,8,8,10,10,11,11,11-heptadecafluoroundecyloxy benzaldehyde

Vita

Sara Ahadi was born on 30th May 1985 in Tehran, Iran. She obtained her Bachelor's in Chemistry from University of Tehran in 2007. She worked on biosensors in her undergraduate research and published two papers in journal of Biophysical chemistry (2008, Vol. 134) and Analytical Letters (2008, Vol. 41). She decided to continue her graduate studies in U.S and finished her Master's in Chemistry at Virginia Commonwealth University.