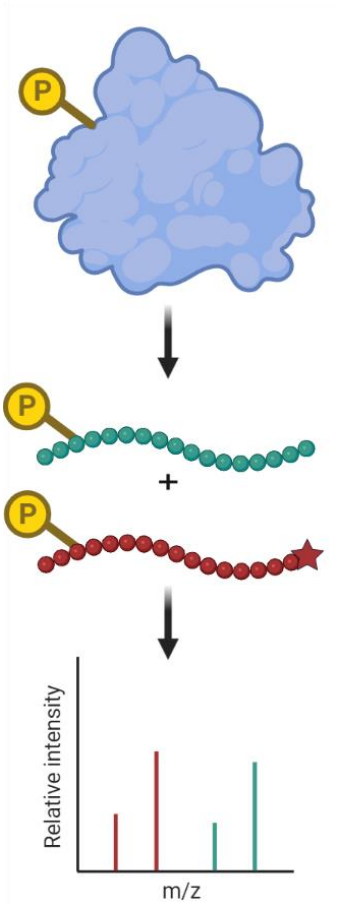
# *IN VITRO* TRANSLATION OF PHOSPHOPEPTIDES AS QUANTIFICATION STANDARD




*Master's Major Research Project | Drug Innovation*

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# Abstract

Phosphorylation is one of the most prevalent post-translational modifications and is involved in multiple biological processes. Several methods have been developed in the field of Mass Spectrometry-based phosphoproteomics to investigate the phosphoproteome. However, quantification in targeted proteomics is still facing challenges in synthesizing isotope-labeled peptides as standards for targeted proteomics. The existing methods to synthesize these can be very expensive and labour intensive. For this reason, a new method was investigated to synthesize standards in a flexible way. This method makes use of an in vitro translation system to incorporate stable isotopes in low quantity and to flexibly modify a peptide with phosphate groups. For that, activated esters of phosphorylated L-serine, L-threonine, and L-tyrosine were synthesized and shown to be acylated on tRNA by use of catalytic RNA called Flexizymes. The incorporation of these amino acids was broadly confirmed by translating green fluorescent protein via stop codon reprogramming. Additionally, reprogramming was also tested by translation a peptide of Tuberous Sclerosis complex 2 via stop codon suppression and tRNA suppression with an antisense oligonucleotide, but no phosphorylated product could be confirmed yet. However, multiple tests and adjustments can still be performed to be able to fully use this method.

# Layman's abstract

After proteins are synthesized from mRNA, they may undergo post-translational modifications to form the complete protein. These modifications are important in many biological processes like cell signalling. One of these modifications is called phosphorylation, which is the addition of a phosphate group. The addition and removal of this group is a very abundant process in cells and is also involved in multiple mechanisms. It is therefore a very interesting field where a lot of research is being performed. For example, it has been found that a dysregulation of this process can be a marker for certain diseases. In Alzheimer's disease it is found that an upregulation of this process can represent a sensitivity towards the disease.

A field that specifically focuses on this modification has made some great advancements in the development of methods to discovery these modifications. However, to date only a small fraction of the phosphorylation sites and their mechanisms have been identified. An approach to uncover these mechanisms is by quantifying proteins with this modification. However, reference peptides with phosphate modifications are essential for this process and the existing synthesis methods have been labour-intensive and expensive. A more convenient and flexible method for the synthesis can therefore facilitate the research in this specific field. Thus, this thesis will describe and test a less labour-intensive and more cost-effective method to synthesize these standards.

This method uses a system that includes all components necessary to transcribe and translate DNA into proteins but without the actual cell. This enables us to modify the system to be able to synthesize products in a way that is normally not possible within a cell. For example, amino acids that are not one of the 20 fundamental amino acids can be used. Phosphorylations are normally performed after the translation. But by using this method, amino acids with those phosphate groups already attached can be incorporated during translation. Thus the first step was to synthesize amino acids with a phosphate group attached. When these were completed, they were tested on their ability to be incorporated in translation. Unfortunately, no data could validate that the amino acids were incorporated. Multiple factors could have influenced these results. But further tests will enable us to make modifications to the system to be able to apply this method.

# Table of Contents

# List of abbreviations

| *Abbreviation* | *Explanation* |
|---|---|
| 5F Phe | 5-fluoro phenylalanine |
| ABT | 4-[(2-aminoethyl)carbamoyl]benzyl thioester |
| ACN | Acetonitrile |
| AcOH | Acetic acid |
| aFx | Amino Fx |
| Boc | tert-Butyloxycarbonyl |
| BPB | Bromophenol blue |
| BSA | Bovine serum albumin |
| CME | Cyanomethyl ester |
| DBE | 3,5-Dinitrobenzyl ester |
| DCM | Dichloromethane |
| dFx | Dinitro Flexizyme |
| DIC | N,N'-Diisopropylcarbodiimide |
| DIPEA | N,N-Diisopropylethylamine |
| DMF | Dimethylformamide |
| DMSO | dimethyl sulfoxide |
| DNB-Cl | 3,5-Dinitrobenzyl chloride |
| EA | Ethyl acetate |
| EDTA | Ethylenediaminetetraacetic acid |
| EF-Tu | Elongation Factor Tu |
| eFx | Enhanced Flexizyme |
| $Et_2O$ | Diethyl ether |
| $Et_3N$ | Triethylamine |
| FAM | 6-carboxyfluorescein |
| Fmoc | Fluorenylmethyloxycarbonyl |
| Fx | Flexizyme |
| HEPES | (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) |
| HPLC | High Performance Liquid Chromatography |
| MeOH | Methanol |
| NaOAc | Sodium acetate |
| PE | Petroleum ether |
| pSer | Phosphorylated serine |
| pThr | Phosphorylated threonine |
| pTyr | Phosphorylated tyrosine |
| PURE | Protein synthesis Using Recombinant Elements |
| RF1 | Release factor 1 |
| RF2 | Release factor 2 |
| RF3 | Release factor 3 |
| SILAC | Stable isotope labeling by amino acids in cell culture |
| SPPS | Solid phase peptide synthesis |
| TIPS | Triisopropylsilane |

| *Amino acids* | *Three-letters code* | *One-letter code* |
|---|---|---|
| Alanine | Ala | A |
| Arginine | Arg | R |
| Asparagine | Asn | N |
| Aspartic acid | Asp | D |
| Cysteine | Cys | C |
| Glutamic acid | Glu | E |
| Glutamine | Gln | Q |
| Glycine | Gly | G |
| Histidine | His | H |
| Isoleucine | Ile | I |
| Leucine | Leu | L |
| Lysine | Lys | K |
| Methionine | Met | M |
| Phenylalanine | Phe | F |
| Proline | Pro | P |
| Serine | Ser | S |
| Threonine | Thr | T |
| Tryptophan | Trp | W |
| Tyrosine | Tyr | Y |
| Valine | Val | V |

# Introduction

Protein phosphorylation is involved in multiple biological processes such as protein folding, signal transduction, and cell cycle regulation (1). It is one of the most abundant post-translational modifications as approximately 2% of the eukaryotic genome encodes protein kinases and phosphatases and it is estimated that 30 to 50% of proteins are to be phosphorylated at some point (2). A dysregulation in this modification has been shown to be a marker in diseases (3). In Alzheimer's disease it is found that upregulated phosphorylation of the microtubule-associated protein tau can represent sensitivity towards the disease (4,5). Mutations in kinases, enzymes that catalyze phosphorylation, often lead to an increase or decrease in function which can cause cancer like leukaemia and non-small cell lung cancer (6). Therefore a lot of effort is being put into identifying phosphorylation sites and functions. But to date, only a fraction of these functions has been identified.

Phosphoproteomics is focused on identifying these phosphosites (7). The methods used for these discoveries are enrichment strategies and mass spectrometry analysis. Enriching of phosphopeptides allowed for better detection via mass spectrometry. Any interesting discoveries are then most likely further characterized by quantifying these phosphopeptides to interpret possible mechanisms.

One of the most well-known methods for quantification is stable isotope labeling in mass spectrometry (3,8,9). With this method, peptides of interest are quantified by using an internal standard. This standard is the same peptide but with a stable isotope incorporated. The sample of interest is spiked with a known concentration of this standard and the mixture is analysed by LC-MS/MS. The unknown amount of peptide in the sample can be calculated by comparing the peak areas which can be separated on their mass difference. But for this method, the target peptide with phosphates will need to be synthesized with a stable isotope.

## Synthesis strategies

One of the most general synthesis methods is Solid Phase Peptide Synthesis (SPPS) in which an amino acid is bound to a solid support and elongated one amino acid at a time as mentioned in Figure 1 (10,11). To add modifications to the peptide, the amino acid building blocks can be adjusted to already have these incorporated. For example, stable isotopes can easily be introduced by using an isotope-labeled amino acid as a building block in the sequence. Phosphate groups can be introduced in the same manner. However, they also need to be modified with protection groups to avoid side reactions. But these can be removed during the cleavage step if chosen correctly. Even though this method can yield the correct peptides quite easily, it can be is quite expensive since it requires a large amount of stable isotope-labeled amino acids.

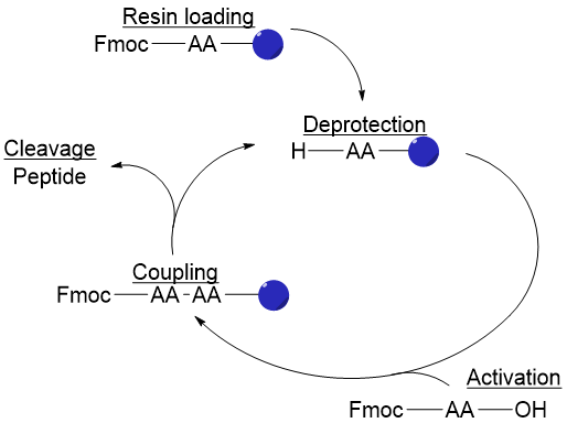



*Figure 1. Schematic representation of Fmoc SPPS. The workflow starts with binding an Fmoc-protected amino acid to the resin. The Fmoc is then removed and another activated Fmoc-protected amino acid is added instead. This process is repeated, and the full peptide is then cleaved off the resin.*

Another method is by Stable Isotope Labeling by Amino acids in Cell culture (SILAC) in which peptides are expressed in cells. Stable isotopes can be introduced by exchanging amino acids for their isotope-labeled version in medium (Figure 2). This method can also incorporate phosphate groups but then by protein expression in mammalian or yeast cells since these can naturally post-translate the product. This synthesis is possible at a smaller scale than SPPS and therefore more cost-effective. But there is little control over phosphorylation site (12).

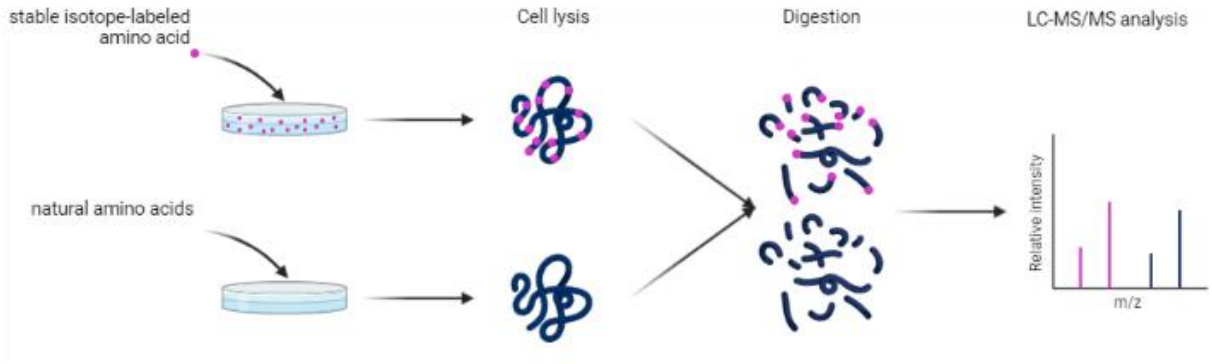



*Figure 2 A schematic overview of SILAC. A known concentration of a stable isotope-labeled amino acid is added to the cell culture and the natural version is omitted. The protein of interest is then expressed with the stable isotope incorporated. The cell lysate with the synthesized standard is then pooled together with the cell lysate of the naturally translated protein of interest. The proteins are enriched, digested, and analysed by LC-MS/MS.*

Fortunately, a more recent synthesis method can synthesize peptides cost-effectively and enables site-specific phosphorylation. This method is called Cell-free synthesis and uses extracts containing all components necessary for transcription and translation (10,13,14). Stable isotopes can be incorporated similarly to the SILAC method, by exchanging amino acids for their isotope version in the translation mixture. This method has not been used yet to synthesize isotope-labeled standards, but has been used for synthesis of recombinant phosphoproteins (14). Phosphoserine (pSer) was incorporated by engineering a tRNA, aminoacyl-tRNA synthetase (aaRS), and elongation factor Tu (EF-Tu) specifically for pSer and stop codon reprogramming (Figure 3). This method can incorporate site-specifically. However, it is very labour intensive to engineer these necessary translational components for every amino acid.

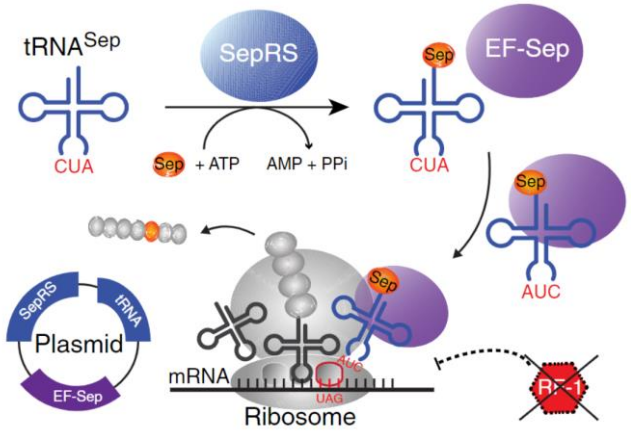



*Figure 3. A schematic overview of pSer incorporation by Oza et al.* (14) *They describe pSer as Sep in this figure. A specifically designed tRNA body for pSer and anticodon CUA is acylated with Sep by a specifically designed aaRS. A specifically designed elongation factor (EF-Tu) to recognize this tRNA leads it to the ribosome. pSer is then incorporated at the UAG stop codon by suppressing Release Factor 1 (RF1). This method of incorporation by stop codon is elaborated further in a later section.*

## The flexizyme

In this thesis a more flexible method to incorporate phosphoamino acids was tested. With use of the Flexizyme (Fx), any amino acid can be introduced site-specific and with low effort. The flexizyme is a flexible tRNA acylation ribozyme capable of charging tRNAs with non-canonical amino acids. Currently three versions of flexizymes are available and each with their own requirements and benefits (Figure 4): dinitro-Fx (dFx), enhanced Fx (eFx), and amino Fx (aFx) (15,16). For amino acids containing aromatic side chains, eFx is best fitting which requires installation of a cyanomethyl ester (CME) on the amino acid. This flexizyme is also the most efficient in acylating tRNA. For those amino acids without aromatic side chains, dFx is best fitting which requires a dinitrobenzyl ester (DBE). And lastly, for amino acids with poor solubility in water, aFx is best which requires a 4-[(2-aminoethyl)carbamoyl]benzyl thioester (ABT). These flexizymes are able to acylate amino acids onto tRNAs by only recognizing the last 3 bases at the 3' end of a tRNA and thus can acylate any tRNA without the influence of the anticodon or tRNA body (17). This acylated tRNA can then be added to the translation mixture to incorporate the amino acid site-specifically. Thus this method can incorporate any amino acids with modifications like a phosphate group. But to use this technique for the synthesis of phosphopeptides, it will require the synthesis of amino acids with a phosphate group in the side chain and an activated ester at the C-terminus.

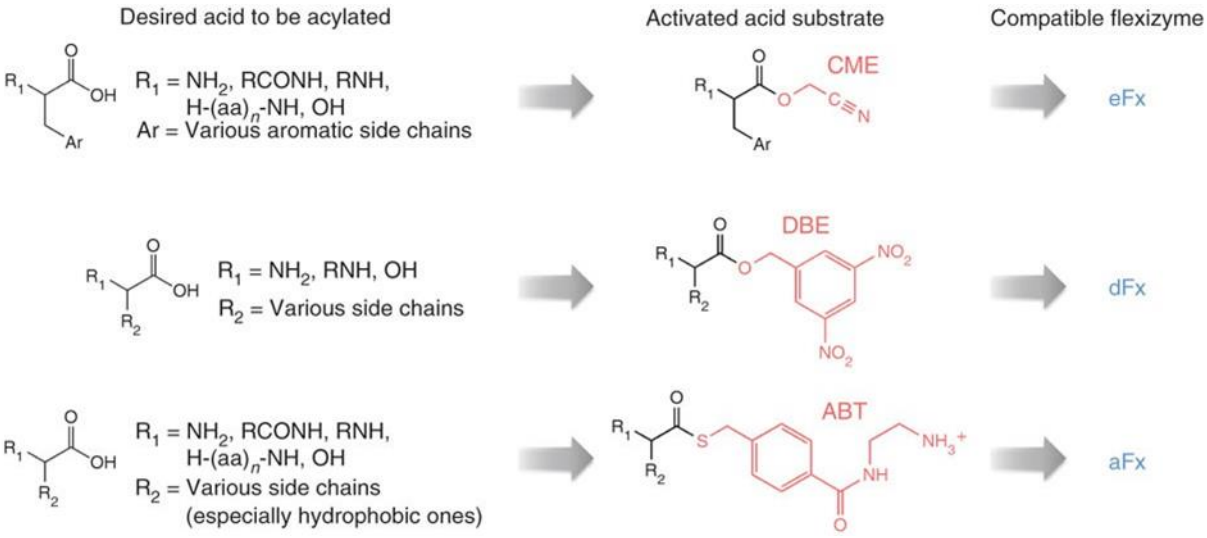



*Figure 4. Overview of the flexizymes with appropriate combination of active group and amino acid substrate. Figure from Goto et al.* (15)

## Quantification

A fitting synthesis strategy has been selected for testing. But to use a phosphopeptide as quantification standard, the amount of labeled peptide needs to be determined. Several biophysical and biochemical methods exist as well for this quantification. For example, a common method is via UV absorption at 280 nm (18). The molar absorption coefficient could be estimated by using a formula. However, this method can only be used on purified peptides to avoid interference of other components. Other methods like a Bradford assay also require a sample of labeled peptide to be modified and thus lowers the yield of the synthesis.

# Indirect quantification method

But a solution for this quantification problem has been published by two groups and this method will be tested in this thesis. Narumi *et al.* and Xian *et al.* both describe the synthesis of a peptide consisting of three elements with stable isotopes by the Protein synthesis Using Recombinant Elements (PURE) system (10,13,19). These elements are the peptide of interest, a purification tag, and a quantification peptide (Figure 5). The purification tag was used to enrich the complete peptide from all the translation components. Xian *et al.* chose for a strep-tag and Narumi *et al.* for a FLAG-tag. The quantification peptide was used as indirect way of quantifying the peptide of interest. It was chosen to be a peptide that can easily be obtained and quantified (further explained in the following paragraph). Xian *et al.* chose for the Strep-tag to also function as quantification peptide and Narumi *et al.* for a tryptic digest of bovine serum albumin (BSA). The Strep-tag can easily be synthesized by SPPS and quantified by weighing or measuring UV absorbance. The tryptic digest of BSA is commercially available. To incorporate stable isotopes, stable-isotope labeled L-arginine and L-lysine were used in the translation mixture and natural L-arginine and L-lysine were omitted

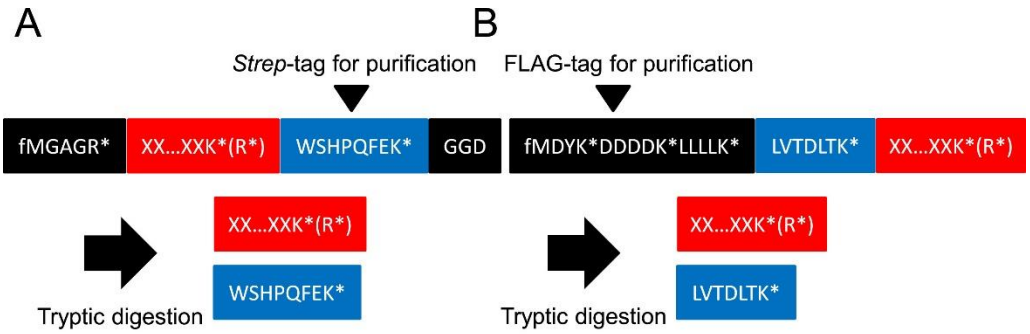



*Figure 5. A summary of the quantification method by Narumi et al.* (10) *A) The peptide design by Xian et al. It contains the peptide of interest in red, the quantification peptide and purification tag in blue and additional components necessary for the trypsin digestion in black. B) The peptide design by Narumi et al. It contains the peptide of interest in red, the quantification peptide in blue, and the purification tag in black.*

After the enrichment step, the complete peptide was cleaved by trypsin digestion. By designing all components with a C-terminal lysine or arginine, the three components could be separated from each other and would each contain a stable isotope. In the trypsin digestion mixture, the three components would be present in equimolar concentrations. Thus the same amount of peptide of interest is present as the quantification peptide. A sample was then spiked with a known concentration of unlabeled quantification peptide that was either synthesized or purchased and afterwards analysed by LC-MS/MS. The peak areas of labeled and unlabeled quantification peptide were used to calculate the concentration of labeled quantification peptide. Indirectly, the concentration of the peptide of interest is then also known as the peptide of interest is present in equimolar concentration as the quantification peptide. Then by spiking the sample of interest with this mixture, and analysing that on LC-MS/MS, the peptide of interest can be quantified.

Both methods can quantify the peptide of interest and only little differences can be found. However, a noticeable difference is the placement of the quantification peptide. Placing the quantification peptide before the peptide of interest could lead to inaccurate quantification. For example, when the translation of the peptide of interest is terminated prematurely in some cases, the quantification peptide and a truncated peptide will then be present in the mixture after digestion. The quantification peptide will then

represent both correctly translated and truncated peptide of interest. Thus the quantification of peptide of interest and quantification is no longer a one-to-one comparison. Especially when a modification is introduced to the peptide of interest by stop codon reprogramming, it could lead to truncated product.

# Investigation

In conclusion, this thesis will investigate a method to synthesize phosphopeptides as a quantification standard in a cost-effective and flexible way with low effort. To start off, modified amino acids were synthesized. Phosphorylation is mostly prevalent on serine, threonine, and tyrosine residues. Therefore, these amino acids will be modified with a phosphate group and an activated ester for the flexizyme. Since serine and threonine do not possess an aromatic side chain, dFx would be the best option for acylation. With that same reasoning, eFx would be a better choice for tyrosine. However, previous attempts to synthesis  sulfated tyrosine with CME have failed(20). Even though phosphates are generally more stable than sulfates, to avoid possibly facing the same problems dFx will also be used for tyrosine acylation. Additionally, acylation of tyrosine by dFx has already been shown in literature.

Next will be testing the amino acids for their ability to be acylated on tRNA by a flexizyme. When acylation of all amino acids has been tested and confirmed, incorporation can be tested by using the PURE system to synthesize reprogrammed peptides. And finally, the quantification by the method of Xian *et al.* can be tested as mentioned in Figure 6 (13).

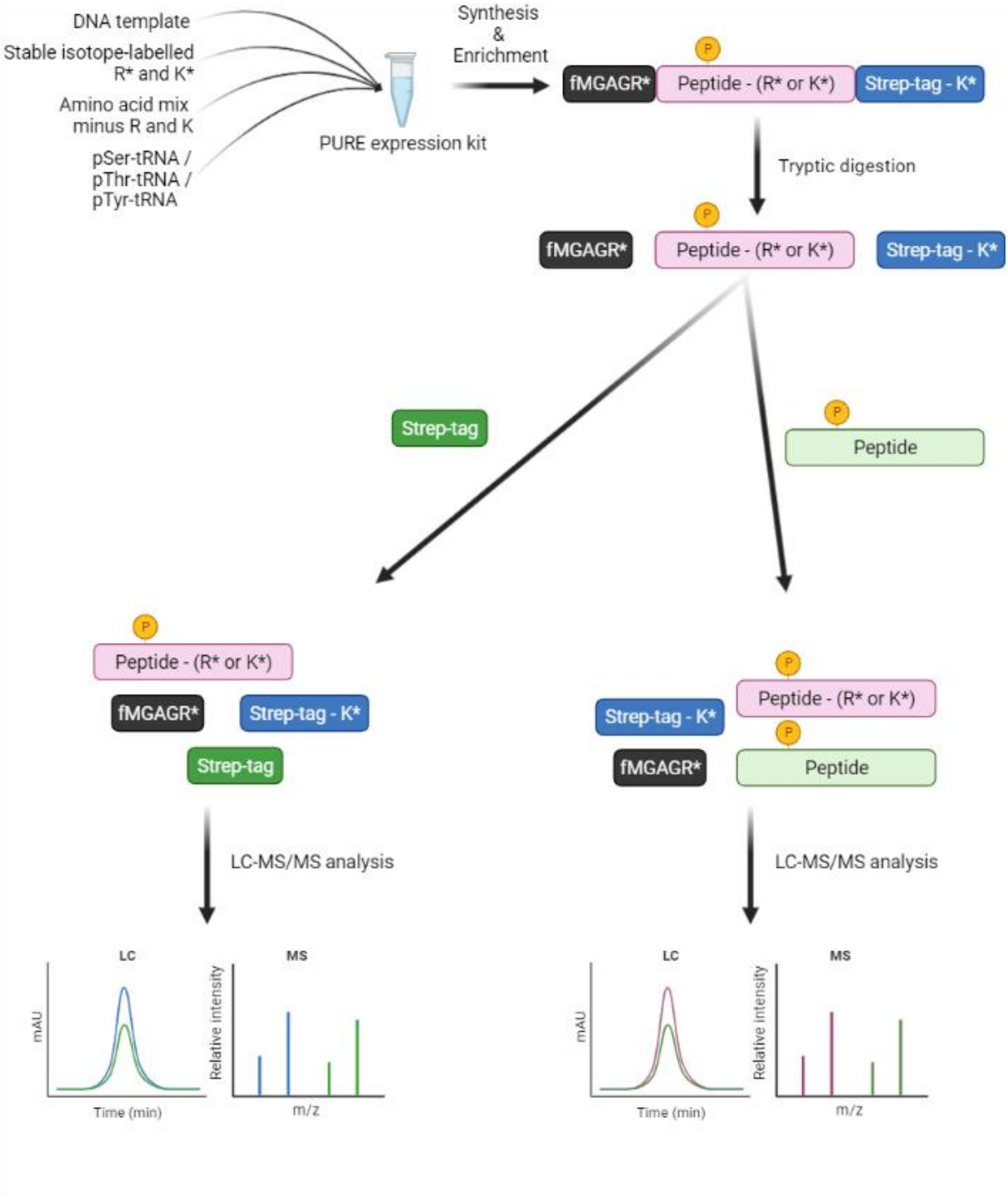
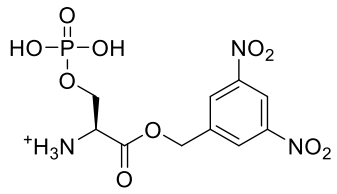


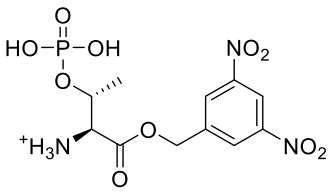

*Figure 6. A schematic overview of the synthesis and quantification method under development in this work. pSer, pThr, or pTyr will be acylated on tRNA and this will be added to in vitro translation mixture using the PURE system. The peptide design will be comparable to the peptide of Xian et al. The concentration of quantification peptide will first be determined with unlabeled Strep-tag. Afterwards, the concentration of the target peptide can be quantified. This figure was created with BioRender.*

# Results

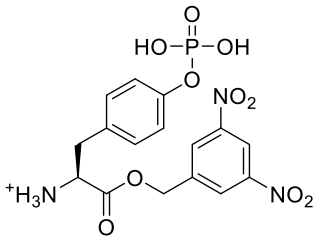
## Synthesis



phosphoserine-dinitrobenzylester
(H-pSer-DBE)



phosphothreonine-dinitrobenzylester
(H-pThr-DBE)



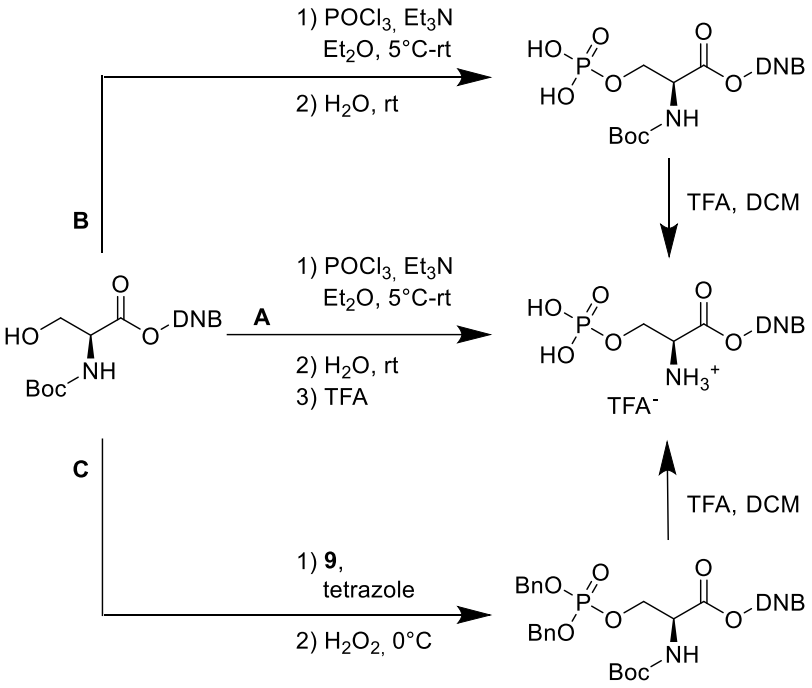
phosphotyrosine-dinitrobenzylester
(H-pTyr-DBE)

*Scheme 1. The three target structures.*

The synthesis of phosphorylated serine, threonine, and tyrosine will be described in this section. The amino acids are modified with a DBE group as activating ester for the flexizyme and a phosphate group modification attached to the hydroxyl group (Scheme 1).

### H-pSer-DBE

Boc-protected Serine with DBE activation was already synthesized previously (11). This compound was synthesized by first installing a Boc protection group on the free amine, followed by installing the DBE component by using 3,5-dinitrobenzyl chloride (DNB-Cl) in a nucleophilic substitution reaction. This intermediate was stored as a dry yellow solid at -20 °C for a year. However, $^{1}$H NMR analysis of this intermediate showed no degradation during storage.

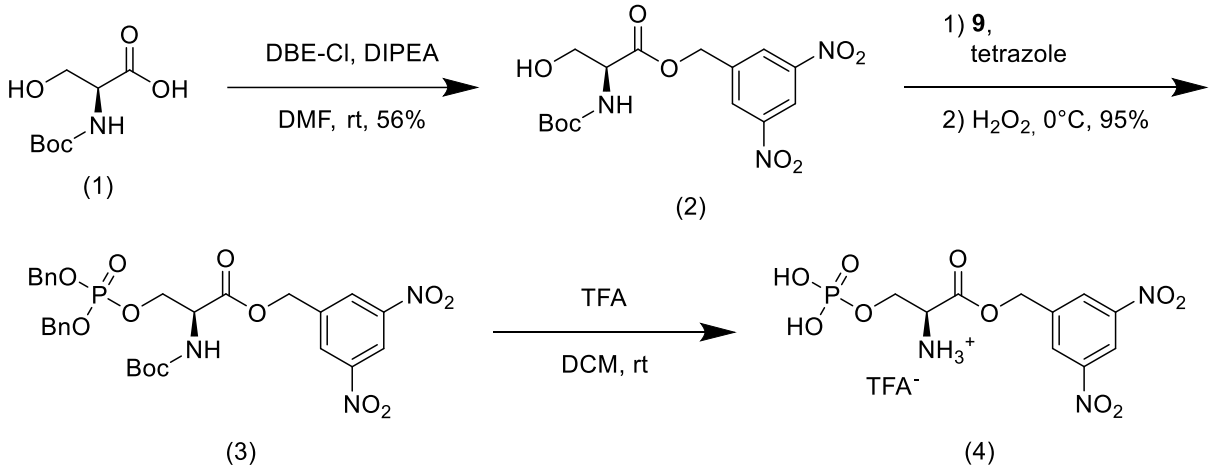



*Scheme 2. A scheme of the phosphorylation attempts for pSer. Synthesis route A: Phosphorylation by $POCl_3$ and directly followed by the removal of the Boc-protection. Synthesis route B: Phosphorylation by $POCl_3$, followed by a purification step before continuing with the removal of the Boc-protection. Synthesis route C: Phosphorylation by **9** (dibenzyl diisopropylphosphoramidite), followed by a purification step before continuing with the removal of the benzyl- and Boc-protections.*

The phosphorylation step was first attempted with phosphorous oxychloride ($POCl_3$) as mentioned in Scheme 2A. Full consumption of the starting material was observed by Thin Layer Chromatography (TLC) after 30 minutes. In this step, a phosphate with chlorides instead of hydroxyls was attached to the hydroxyl of serine. Thus, the reaction was quenched with $H_2O$ to substitute all chlorides for hydroxides. The formed HCl was neutralized with $Et_3N$. Without purifying the reaction mixture, Boc deprotection with TFA was started. However, the product could not be extracted out of the aqueous layer during the work-up afterwards. Lyophilizing the product and desalting by a C8 column were tried, but without success. It is most likely that the product was too polar because of the free amine and phosphate group.

The intermediate was synthesized again as described and phosphorylation by $POCl_3$ was tried again as described in Scheme 2B. This time, a work-up was performed to isolate the Boc-protected phosphorylation product. This intermediate is less polar than the deprotected version and thus should promote isolation via extractions. But also this intermediate could not be extracted from the aqueous layer.

To solve the purification problem, phosphorylation with a phosphoramidite was performed as mentioned in Scheme 2C. This approach installed a phosphite with benzyl protections. The product was oxidized afterwards and the work-up was this time successful. The intermediate was purified by flash column chromatography. A Boc removal from the amine and benzyl removals from the phosphate were performed in parallel with 25% TFA in DCM and finalized the synthesis of pSer with a total yield of 53% (Scheme 3).




*Scheme 3. Synthesis scheme for pSer. The yield for the final deprotection step could not be calculated due to crystallization with an unknown amount of water and TFA.* ***9****: dibenzyl diisopropylphosphoramidite*

## H-pThr-DBE

The synthesis of phosphothreonine (pThr) was already performed and documented last year as depicted in Scheme 4 (11). The DBE moiety was installed in the same manner as in the synthesis of pSer. But for threonine, the phosphate group could be installed via $POCl_3$. However, the Boc group was removed during this step by the formation of HCl since an insufficient amount of base was used. To completely remove all Boc protections, TFA was added. This also caused a salt exchange from HCl salt to TFA salt. This enabled isolation of pThr via extraction due to the decrease polarity. It also benefited that threonine is slightly less polar than serine.

The final product was purified by flash column chromatography and High-Performance Liquid Chromatography (HPLC) and the fractions with product were lyophilized. However, the product was still within solution when the COVID-19 pandemic started. Because the facilities would be closed for indefinite time, the product was stored at -20 °C in an aqueous solution. Unfortunately, this caused the activated ester to hydrolyse partially. This was confirmed by NMR and LC-MS as shown in Figure 7. Based on the integrals of the NMR spectra, roughly 40% of the product was degraded.

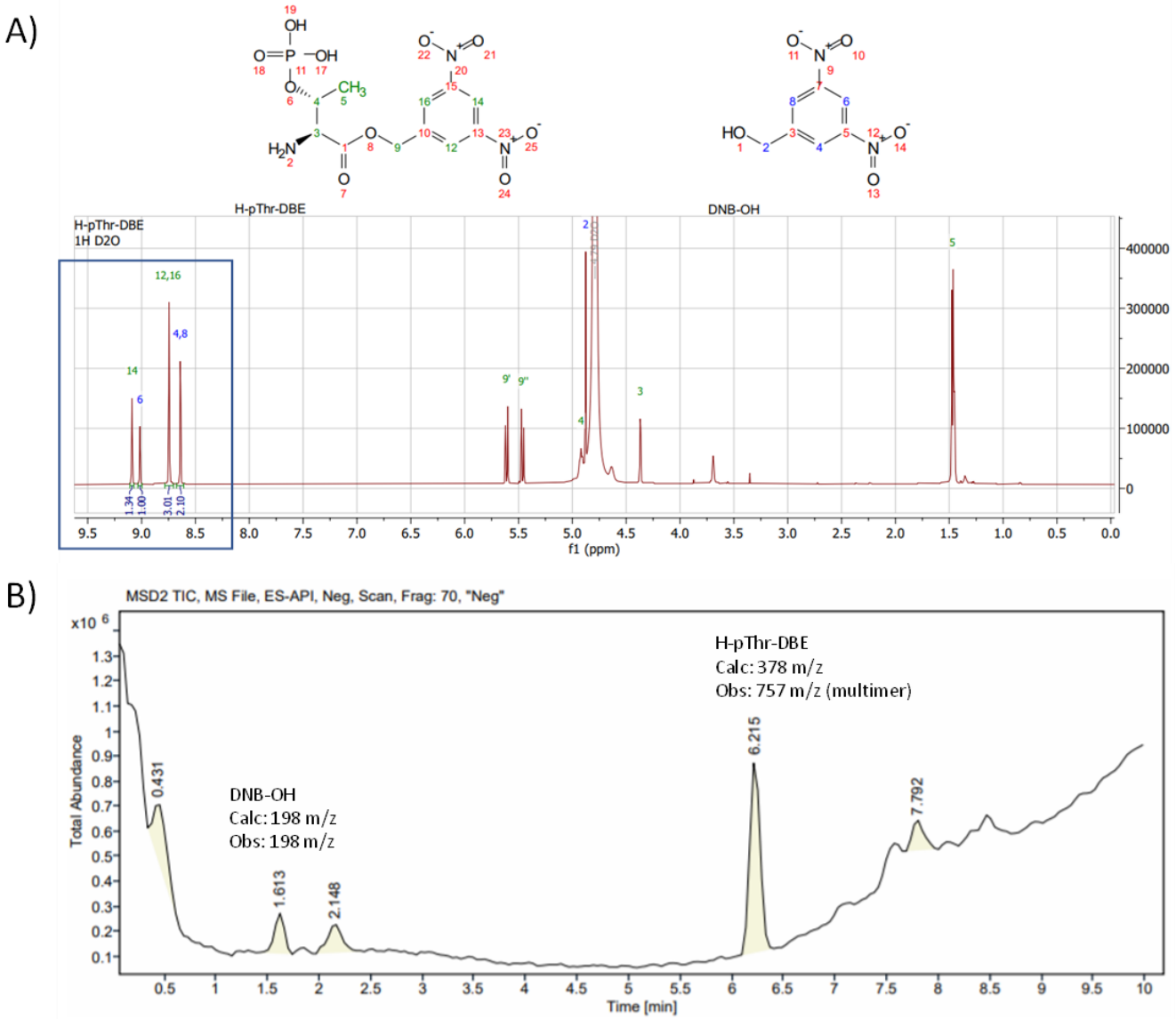
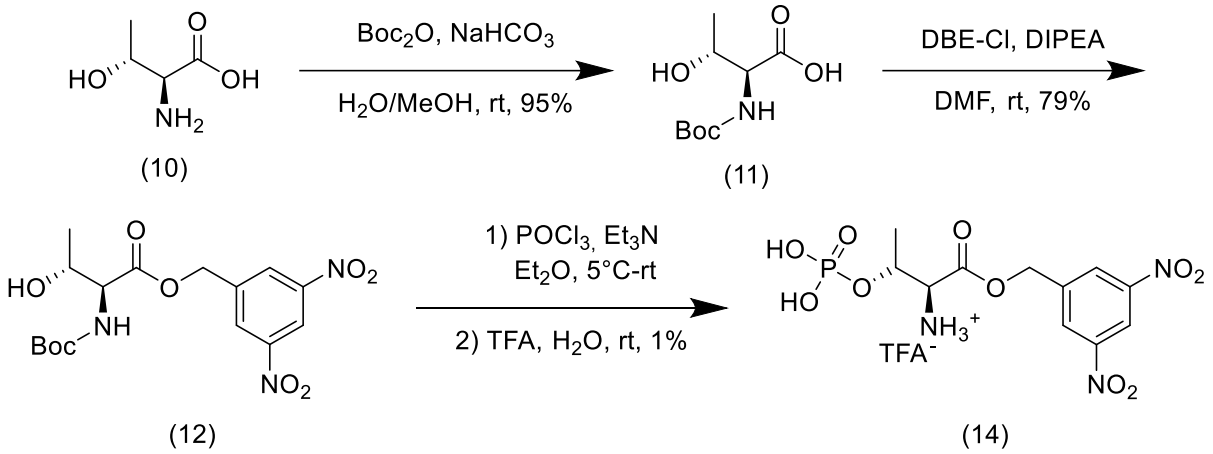



*Figure 7. Analyses of pThr after 1 year. A) $^{1}$H NMR spectra showing peaks for both H-pThr-DBE and DNB-OH. The peaks corresponding to the DBE moiety are emphasized with a box. The amount of degradation was estimated by calculating the ratios of DBE peak integrals. The ratios 1.3:1 and 3:2.1 (pThr : DNB-OH) were calculated and averaged 1.4:1. B) LC-MS spectra for negative ionization labeled with Rt, m/z observed, and corresponding compound name. (multimer formation of pThr* (21)*)*

After lyophilization, the yield could be calculated. Due to a bug in the HPLC computer system, a large amount of the injection was thrown in the waste. Thus only a crude yield of 2,0 mg was achieved. However, this crude could still be used to test acylation. The hydrolysed product and DNB-OH should not interfere with acylation as both cannot be acylated efficiently by dFx.



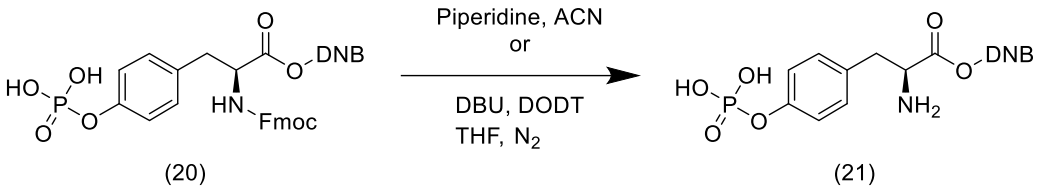

*Scheme 4. Synthesis scheme for pThr as it was synthesized in 2020* (11).

The product was synthesized again as described after the acylation test but with some minor changes. Boc-protected threonine activated with DBE was synthesized with the same yield. However, the phosphorylation reaction by $POCl_3$ was this time unsuccessful. No conversion was confirmed with TLC and LC-MS, and after a normal work-up, also NMR validated this finding. The two biggest differences between this attempt and the previously successful attempt are the addition of more base and the use of a new stock of $POCl_3$. A small-scale reaction without base was also tested, but only yielded deprotected threonine without phosphate or starting material. The previous stock of $POCl_3$ was most likely to be contaminated with $H_2O$ and partially degraded reagent. As an attempt to mimic this condition, a small drop of water was added in the reaction mixture to partially quench the reaction. On TLC no change was observed. However, LC-MS showed a mixture of starting material and phosphorylated product. This can imply that this reaction is dependent on the ratio $POCl_3$ and base. In the future, phosphorylation via this route could be optimized by testing different bases and ratios. However, a more promising attempt would be by using phosphoramidite as was used for the phosphorylation of pSer. This reaction has been successful for pSer and thus can be carried out without optimalisation steps. Furthermore, this will allow for purification before the deprotection step.

## H-pTyr-DBE

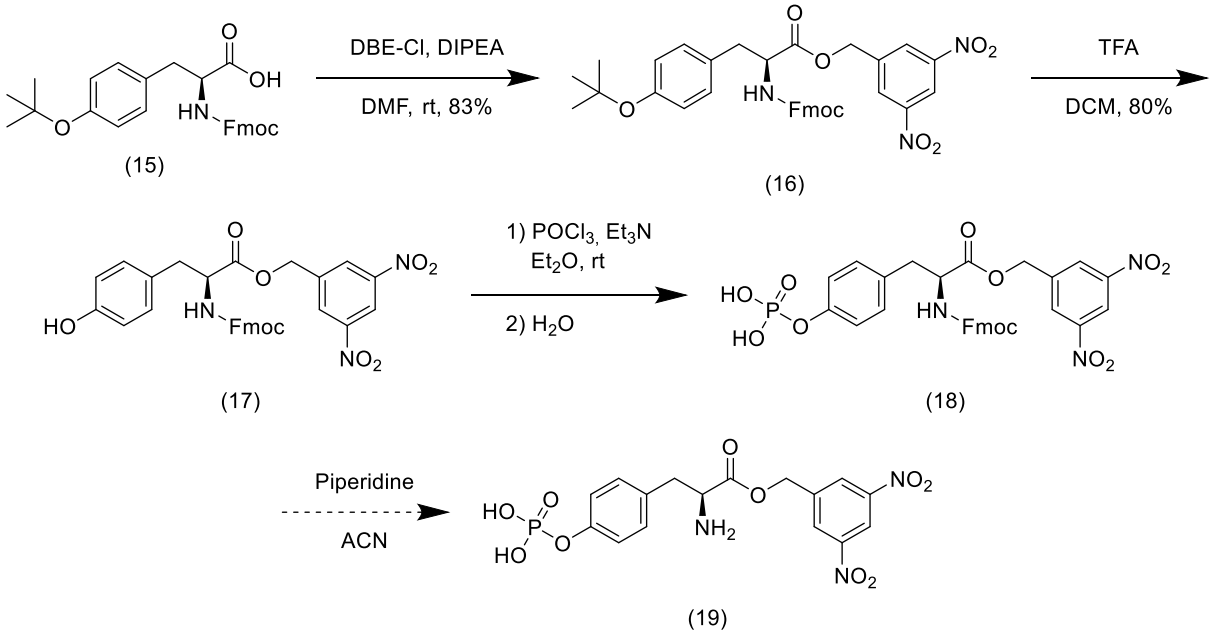
The synthesis of phosphotyrosine (pTyr) started off different than pSer and pThr. Fmoc-protected tyrosine was used as a starting material since an Fmoc protected tyrosine with DBE activation was also necessary for another synthesis. An Fmoc protection instead of a Boc protection should not interfere with the phosphorylation reaction and should therefore also yield phosphorylated tyrosine.

Thus Fmoc protected tyrosine was first modified with a DBE group by DNB-Cl, followed by removal of the *tert*-butyl protection group (Scheme 6). The synthesis of pTyr was continued by phosphorylation with $POCl_3$. In an attempt for optimization, this reaction was carried out in dry atmosphere. However, with these conditions no conversion of the starting material was observed on TLC. The phosphorylation step was performed again without modified atmosphere and yielded the desired intermediate. This shows that a slight contamination by $H_2O$ could benefit the reaction as was also speculated in the pThr synthesis.



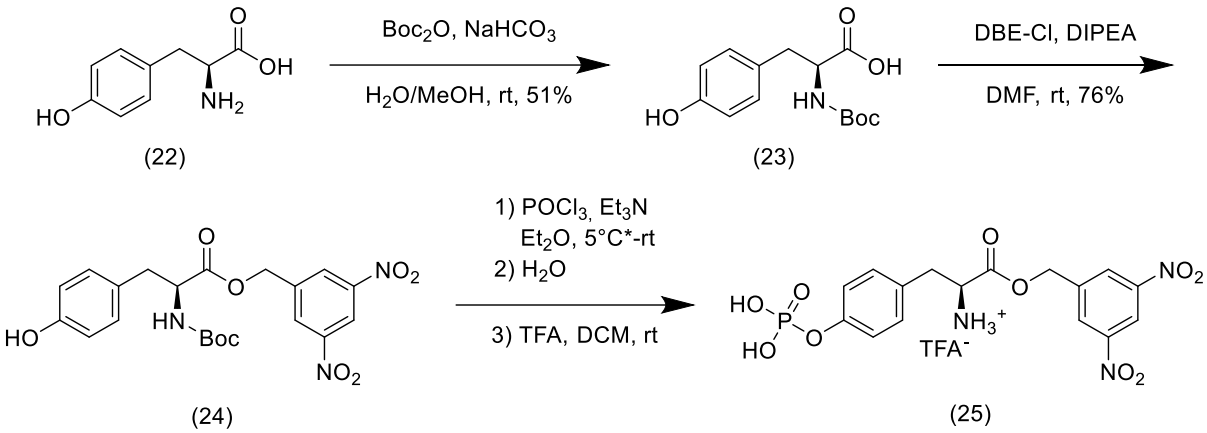

*Scheme 5. Fmoc deprotection attempts for pTyr. Deprotection by piperidine was successful but the product could not be isolated. Deprotection by DBU was unsuccessful.*

The last step was removal of the Fmoc deprotection with piperidine (Scheme 5). Complete conversion was observed with TLC and most of the piperidine was removed on a rotary evaporator. The residue was dissolved in dimethyl sulfoxide (DMSO) and injected on preparative HPLC for purification. However, the product flushed off the column without delay together with DMSO and side products due to residual piperidine and salts. The first fractions were collected and lyophilized. Another attempt to purification was by precipitation from diethyl ether ($Et_2O$). Unfortunately this method was also unsuccessful in purifying the final product as the product was not detected in the precipitate by NMR. Purification by preparative HPLC was tried but was again futile. Deprotection by DBU was also tested, as discussed by Sheppeck *et al.,* but resulted in either dephosphorylation or hydrolysis of DBE (Scheme 5)(22).




*Scheme 6. Synthesis scheme for pTyr via Fmoc protection. The deprotection step was unsuccessful.*

Since the deprotection conditions seemed to hinder the purification of pTyr, an approach with a Boc protection group was tested. A Boc protection was installed on free L-tyrosine, followed by addition of the DBE group with the same procedure as for pSer and pThr. The phosphorylation step afterwards was successful and directly followed by the Boc deprotection with TFA. Via this deprotection, purification by preparative HPLC was possible since the product is more polar after a Boc removal versus an Fmoc removal. This synthesis route gave pure product with a total yield of 61% (Scheme 7).




*Scheme 7. Synthesis scheme for pTyr via Boc protection. The yield for the final deprotection step could not be calculated due to crystallization with an unknown amount of water and TFA.*

# Acylation test

The acylation ability of the amino acids was tested by using a fluorescently labeled oligonucleotide designed by Fujino *et al.* (17) They discovered that the flexizyme can acylate substrates of 4 nucleotides with similar efficiency as the complete tRNA. Before this discovery, aminoacylation was tested by acylating the microhelix. This is a 22-base substrate of tRNA and when this substrate is acylated with an amino acid and analysed on acid-page gel, a shift between acylated and deacylated is visible. However, a long gel had to be run overnight and it was typically not visible for acidic or very small amino acids. Therefore a smaller substrate was necessary. Fujino *et al.* designed a fluorescent-labeled 4-base RNA substrate (4bRNA). The molecular weight of this substrate is a sufficiently lower than the microhelix which enabled the analysis of the acidic amino acids as well (see Figure 8) . Thus this substrate is now preferred over the microhelix. In this work we used a 5 base equivalent due to manufacturer limitations.

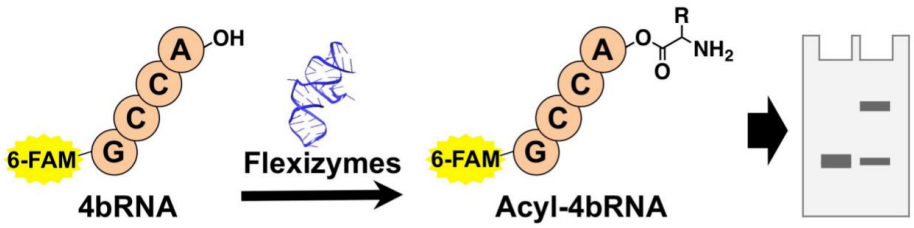
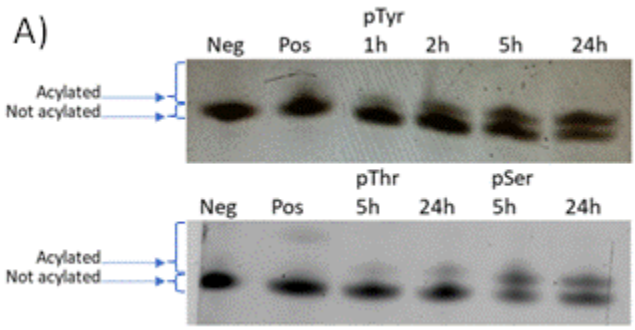



*Figure 8. Schematic representation of the aminoacylation testing with 4bRNA by Fujino et al.* (17) *When an amino acid is acylated on 4bRNA, the mass to charge ratio changes enough to see a separation on gel. Visualization is enabled by the fluorescent label 6-carboxyfluorescein, 6-FAM (λex = 488 nm, λem = 530 nm).*

The efficiencies of pSer, pThr, and pTyr were measured at various time points with a maximum of 24 hours. The samples were analysed using a 20% denaturing acid-PAGE gel. A distinct conversion of mass was visible for pTyr acylation. Acylation of pSer and pThr were not observed during the first analysis but was visible when the samples were allowed to separate further on gel (Figure 9A). The acylation efficiency was estimated by ImageJ and shown in Figure 9B. An acylation efficiency of 10% should yield enough acylated tRNA for translation (23).




B)

| *Aminoacylation efficiency* | pSer | pThr | pTyr |
|---|---|---|---|
| *1 hour* | | | 7% |
| *2 hours* | | | 15% |
| *5 hours* | 49% | 16% | 35% |
| *24 hours* | 43% | 19% | 51% |

*Figure 9. Analysis and quantification of aminoacylation test with 20% denaturing PAGE gel. Samples were taken at different time points. Negative control: DMSO, positive control: A) 20% denaturing PAGE gel with visible shifts. B) Quantification of aminoacylation estimated by densitometry in ImageJ.*

# Translation by PURE

It has been confirmed that all amino acids can be acylated on tRNA, thus the next step is to examine on which tRNA body to use. It has been found that both tRNA and amino acid influence the binding to Elongation factor Tu (EF-Tu) (24). The binding affinity affects the incorporation efficiency during translation. A too strong binding will prohibit the amino acid to release from EF-Tu and a too weak binding will not guide the amino acid to the ribosome. Thus the tRNA body and amino acid need to counterbalance each other for the appropriate binding strength. 19 tRNA bodies were ordered on their affinity for EF-Tu by Dale *et al.* (24) The glutamic acid tRNA body had the strongest binding and the asparagine tRNA body was on the weaker end of the range. Therefore, these two tRNA bodies were assessed on their ability to incorporate the phosphoamino acids in translations. Engineered versions of standard tRNA bodies were prepared by cell-free transcription of the corresponding DNA template. Thus, EnGlu and EnAsn were acylated with the phosphoamino acids.

Another aspect to consider is what method for vacant codon creation will be most productive. Two methods will be discussed in the following sections: stop codon reprogramming and tRNA suppression. To implement these methods, translations of green fluorescent protein (GFP) and a phosphopeptide of Tuberous Sclerosis complex 2 (TSC2) were carried out. TSC2 is a tumour suppressor and activates specific GTPases (25,26). The protein has many phosphosites and one of these is at residue S981.

Reprogrammed GFP translation was used to broadly investigate the incorporation of non-canonical amino acids via fluorescence output. However, this test does not confirm which amino acid is incorporated. Therefore, a phosphopeptide of TSC2 (residues 979-988) was also translated since it could be analysed by an available LC-MS system. The DNA template for this peptide was designed with a strep-tag for quantification and with a TAG, TGA, or AGC codon for reprogramming (Figure 10).

DNA templates

*AGC reprogramming*

ATGAAG TCCATTAGCGTGTCCGAACATGTGGTGCGC TGGTCCCATCCGCAGTTTGAAAAGTAA

*TAG reprogramming*

ATGAAG AGCATTTAGGTGAGCGAACATGTGGTGCGC TGGTCCCATCCGCAGTTTGAAAAGTAA

*TGA reprogramming*

ATGAAG AGCATTTGAGTGAGCGAACATGTGGTGCGC TGGTCCCATCCGCAGTTTGAAAAGTAA

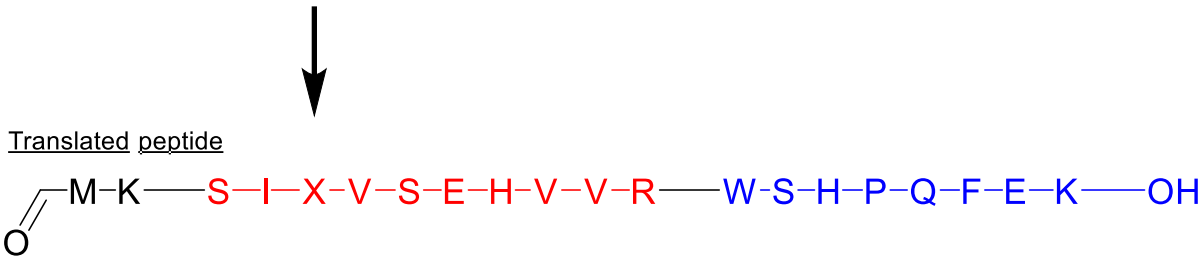



*Figure 10. Peptide design with reprogramming site in the DNA template underscored, and in the peptide represented by X. In red is the target peptide, in blue is the quantification peptide (Strep-tag), and in black the residue peptide.*

## Stop codon reprogramming

During the translation of in bacteria, termination is promoted by the binding of release factors to a stop codon. Two release factors are involved in the recognition of the stop codons; release factor 1 (RF1) recognizes the amber (TAG) and ochre (TAA) codon, and release factor 2 (RF2) recognizes the opal (TGA) and ochre (TAA) codon (27,28). By omitting RF1 or RF2, one of the stop codons is not recognized and thus creates a vacant codon. The ochre codon cannot be vacated since it is recognized by RF1 and RF2.

To test the incorporation of the amino acids by amber codon reprogramming, translations of GFP were executed. A GFP plasmid with a TAG-codon inserted was translated with pSer acylated on either EnGlu or EnAsn. 5-Fluoro phenylalanine (5F Phe) was translated in GFP by use of the amber codon as a positive control for reprogramming. A natural translation of the reprogrammed GFP DNA template with a TAG-codon was carried out as negative control to check for background fluorescence or non-specific codon read-through. This was tested in duplicate. Alongside, GFP wildtype (Wt) was naturally translated to track the translation rate, and a translation without DNA template was also performed to check for any fluorescence that might be produced by the translation components. Next to that, pThr and pTyr were also incorporated to validate extension of this method to other amino acids. These translations were not yet replicated.

A clear difference was visible between the negative control and positive control which means that this method shows enough difference in fluorescence to check for reprogramming (Figure 11). The translation with pSer-EnGlu showed a similar translation level compared to the positive control as were pThr and pTyr on EnGlu as well. However, the translation with pSer-EnAsn showed a translation level that was more comparable to the negative control. It can therefore be concluded that EnGlu is the most appropriate tRNA body for the phosphoamino acids.

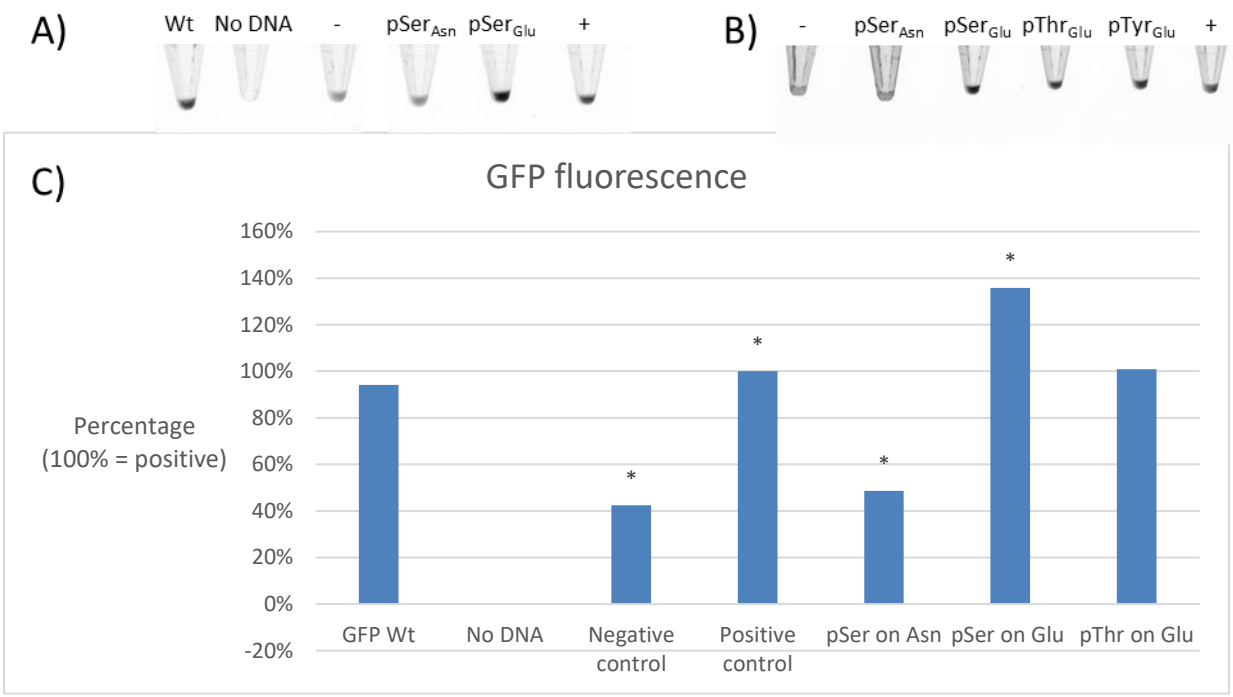



*Figure 11. A and B) GFP fluorescence visualized in Eppendorf vials by an Azure gel scanner (EPI Blue light source, 100 ms exposure). All translation conditions were divided over two translation experiments A and B. Thus negative control, pSer on EnAsn, pSer on EnGlu, and positive control were performed in duplicate. C) A bar graph of the GFP fluorescence compared to the positive control for reprogramming as 100%. The translation mixtures were diluted 1:10 with PBS (1x, pH 8.0) and transferred to a Greiner 96-well white flat bottom plate. The fluorescence was measured with a CLARIOstar plate reader. (ex: 470 – 15, em: 515 – 20, gain: 750) * mean value was taken for the duplicated translations*

To test the incorporation of phosphoserine specifically, the TSC2 peptide with TAG reprogramming was translated with pSer-EnGlu$_{CUA}$. The translation was carried out and a sample was used for trypsin digestion followed by analysis on an orbitrap-QE at the Altelaar group. However, only a non-phosphorylated and partially digested fragment was detected. A phosphoprotein enrichment technique with Fe-column will still be carried out to check if the desired product is present, but in low abundance. Meanwhile, another translation with pSer-EnGlu$_{CUA}$ was executed, purified with a C18 ZipTip, and analysed on the available LC-MS system at the VU (Agilent Single Quadrupole LC/MS). However, also this translation only showed a small amount of dephosphorylated product.

While waiting for results of the phospho-enrichment, enrichment by using MagStrep “type3” XT Beads was also tried. To elude the product from the beads, biotin was used. Unfortunately, it was found during the tRNA suppression test that biotin has the same retention time as the desired peptide on the LC-MS system present. This was found by naturally translating the AGC reprogramming DNA template. Due to the high abundance of biotin, detection of the product was not possible.

## Phosphate stability in the presence of $Mg^{2+}$

It was speculated that the phosphate group was susceptible to $Mg^{2+}$-mediated hydrolysis. It has been mentioned in literature that $Mg^{2+}$ catalyses the hydrolysis of phosphodiesters by forming a complex with phosphorus (29–31). This mechanism was mainly investigated in nucleotides due to its role in DNA and RNA cleavage. (Figure 12)

To test the possibility of hydrolysis during the aminoacylation of tRNA, pSer was incubated with $H_2O$, HEPES-KOH buffer and $MgCl_2$ for 3 days on ice in the dark. The components were present in the same concentrations as it would be during the aminoacylation. The sample was analysed on LC-MS after 3 days and only phosphorylated serine could be found. Thus it can be assumed that only phosphorylated serines are acylated onto the tRNAs.

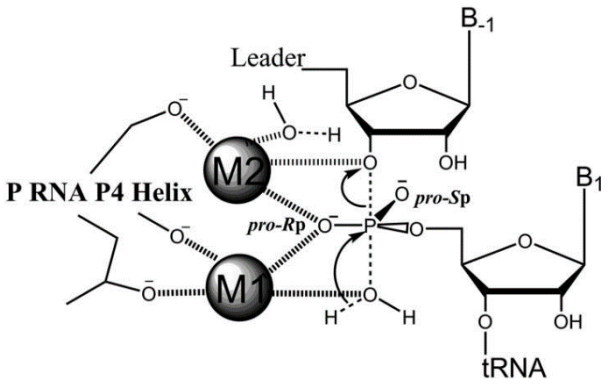



*Figure 12. A possible mechanism for phosphodiester bond hydrolysis by Howard et al.* (31)

## tRNA suppression

The other method for vacant codon creation that was investigated, was by tRNA suppression. Cui *et al.* designed an oligonucleotide that binds specifically to the anticodon and variable loop of only $tRNA^{Ser}GCU$ and thereby inactivate it for translation (Figure 13) (32). By inactivating this tRNA, the AGC codon becomes vacant for reprogramming. Any tRNA with a different variable loop can be used to insert a non-canonical amino acid on this codon, without removing serine from the pool of translatable amino acids (provided the DNA template has a different codon for serine).

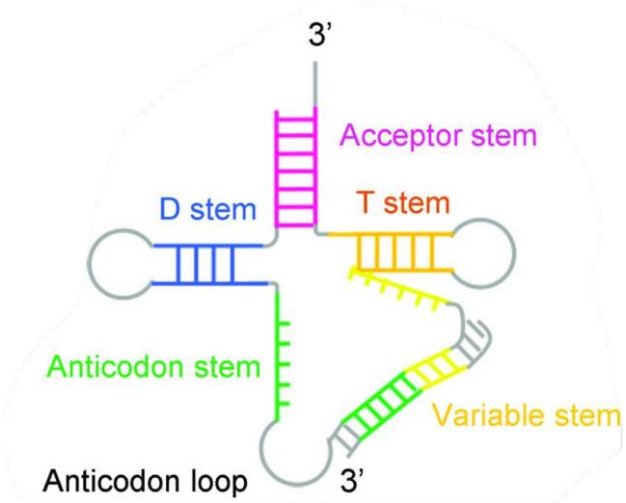



*Figure 13. A schematic representation of the proposed mechanism of antisense oligonucleotide by Cui et al.* (32) *The oligonucleotide binds partially the anticodon and variable loop.*

AGC codon reprogramming was tested by translating TSC2-AGC at 5 µL scale. As a positive control for the translation system, the DNA template was translated without suppressor. The reaction mixture was first purified through a ZipTip and afterwards analysed with LC-MS. The positive control analysis showed formation of product as can be seen in Figure 14. Translation with pSer-EnGlu$_{GCU}$ and with $tRNA^{Ser}GCU$ suppressor was also analysed but did not show product. To check if reprogramming by this method is

productive with this DNA template, translating with 5F Phe-EnGlu$_{GCU}$ was also tested. However, this product was also not found on LC-MS. This either means that reprogramming by this method does not work for this DNA template, or the analysis method is not optimal for reprogrammed products (most likely due to low abundance of product). For the latter problem, enrichment methods could be tested.

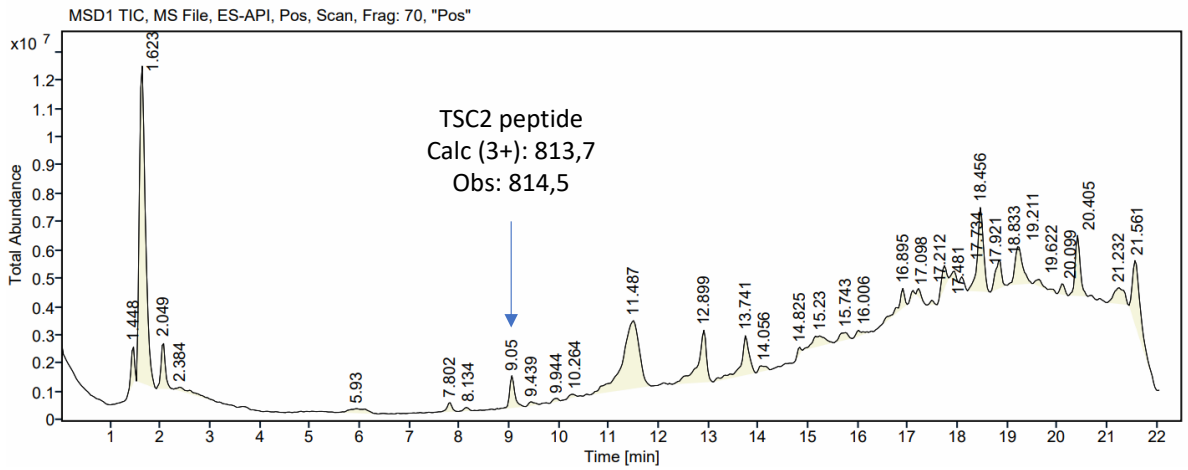



*Figure 14. LC-MS spectra for positive ionization labeled with Rt, m/z observed, and compound name.*

# Conclusion

The synthesis of phosphoserine and phosphotyrosine were completed. Together with the phosphothreonine previously synthesized, the compounds could be tested on their ability to be acylated by dFx. And it was demonstrated that the amino acids could be efficiently acylated in 5 to 24 hours. With this information, tRNAs were prepared and translations were tested. The translation of GFP showed us that pSer, pThr, and pTyr could be incorporated with a EnGlu body sequence.

Unfortunately, no conclusive data can be shown on the incorporation of phosphoamino acids specifically. This could be due to detection problems caused by the LC-MSD XT system used since the detection limit of this system is not optimal for analysing these mixtures. To investigate if this could be the problem, a translation of the TSC2 peptide by TAG reprogramming was sent to the Altelaar group to be analysed on an orbitrap-QE. However, up until now, only dephosphorylated product was found. It was speculated that the phosphate could be lost at the aminoacylation step due to the high concentration of $Mg^{2+}$. However, this was ruled out by testing the stability of pSer under the same conditions as during the aminoacylation. Another possibility is that the translation system has components that could dephosphorylate the product or tRNA. Since a commercial expression kit was used, it is unknown which proteins were present, including perhaps contaminating phosphatases. This could possibly be investigated by using a homemade expression system of which all components are known or including phosphatase inhibitors.

There is also a possibility that EF-Tu is not accepting pSer. Park *et al.* was able to incorporate pSer by engineering a tRNA, aaRS and EF-Tu specifically for pSer (33,34). An engineered EF-Tu was necessary because the original EF-Tu used was not recognizing the engineered tRNA. EnGlu and EnAsn have been used before in reprogramming successfully. Thus EF-Tu should be able to recognize this tRNA. So, what could be investigated, is the binding strength of pSer to EF-Tu in order to validate if EF-Tu is the limiting factor for the translation.

There is still a possibility that phosphopeptides were synthesized but not detected. Phosphoenrichment methods can be applied to check for phosphorylation. A method that was going to be performed at the Altelaar group, was enrichment via an Fe-column. The enriched sample can then still be analysed on LC-MS. If this method is successful, it can also be investigated if this method can be used to enrich the full peptide from the translation components. Another method to just detect any phosphorylation, is by blotting the translations with phosphospecific antibodies. Antibodies for pSer, pThr, and pTyr are commercially available.

And lastly, if incorporation of phosphoamino acids by this method is unlikely, it can also be accomplished by using modified tRNA, aaRS, and EF-Tu. These have already been engineered for pSer and pTyr, but not for phosphothreonine. The pSer system may accept pThr, but synthesis of a peptide consisting of both pSer and pThr will then not be possible. Moreover, the method with the flexizyme is more flexible with new modifications. If the flexizyme is replaced for the engineered acylation elements, it will become very labour-intensive to incorporate new modifications.

In the end, the goal is to have a system to synthesize isotopic-labeled peptides with site-specific phosphorylation for quantification. A lot of preparation and experiments have already been performed and provided a lot of that. Although these tests were unable to confirm this method, multiple options for follow-up investigations can still validate this method.

# Experimental methods

## Materials and equipment

Reagents and solvents were obtained from chemical suppliers as Sigma-Aldrich, VWR and Thermo Scientific. TLC with aluminium backing (SiliCycle 200 µm with F-254 indicator) was used to monitor the amino acid syntheses and the compounds were visualized by both UV absorption (254/365 nm) and staining with ninhydrin followed by heat. Flash column chromatography made use of silica gel (ZEOprep 60/40 – 63 µm) for purification.

$^{1}H$, $^{13}C$, and $^{31}P$ NMR spectra were obtained from a Bruker Biospin NMR 500 MHz or Varian 400 MHz / 600 MHz. Preparative HPLC was performed by using an Agilent prep LC with a Phenomenex (USA) Gemini C18 column (250x21.20 mm, 10 mm), buffer A (0.1% TFA in $H_2O$), and buffer B (0.1% TFA in ACN). Analytical LC-MS was performed by using an Agilent single quadrupole (SQ) LC/MSD XT with a Poroshell 120 EC-C18 (120 Å, 4.6 x 100 mm, 2.7 µm), buffer A (0.1% FA in $H_2O$), and buffer B (0.1% FA in ACN). The spectra are collected in the Supplementary data section.

Gels were analysed by Bio-Rad Gel Doc XR+ or Azure biosystems C200 gel scanner. 96-well plates were analysed by a CLARIOstar microplate reader from BMG Labtech. PCR was performed by an Edvotek EdvoCycler Jr. Personal PCR Machine and a NanoDrop 2000 Spectrophotometer from Thermo Scientific was used to measure the nucleic acid concentration.

The flexizyme dFx and the tRNAs were prepared according to literature (15). Briefly, the standard protocol consists of primer extension by PCR, *in vitro* transcription by T7 RNA polymerase, and a 10% denaturing PAGE gel purification.

## Aminoacylation efficiency test

### Sample preparation

In a tube were added $H_2O$, HEPES-KOH (pH 7.5, 50 mM), dFx (25 µM), and FAM 5b tRNA (25 µM). The reaction mixture was heated for 2 min at 95 °C, followed by 5 min at rt. $MgCl_2$ (300 mM) was added and the mixture was again left to rest for 5 min at rt. The mixture was chilled on ice before adding the amino acid (2.5 mM), and the complete mixture of 10 µL was incubated on ice in the dark. L-propargylglycine was used as positive control and FAM 5b tRNA in water was used as negative control. H-pSer(P)-DBE, H-pThr(P)-DBE, and H-pTyr(P)-DBE were tested.

Loading buffer consisted of formamide (23%) , NaOAc buffer (pH 5.2, 0.1 M), EDTA (6 mM), BPB (0.04%), and $H_2O$ in a final volume of 100 µL. After 1h, 2h, 5h, and 24h, 2 µL of the reaction mixture was taken and quenched with 2 µL loading buffer. The samples were stored at -20 °C until all samples were taken.

### Gel analysis

The 20% denaturing PAGE gel was prepared by first dissolving urea in acrylamide (20%), NaOAc buffer (pH 5.2, 50 mM), and $H_2O$. After the urea was fully dissolved ammonium persulfate (APS) (0.1%) and tetramethylethylenediamine (TEMED) (0.08%) were added, and the mixture was immediately poured in a gel casting (1.5 mm thick) and left at room temperature to set.

The samples were loaded on the gel, 2 µL of each sample. The gel was kept in 50 mM NaOAc (pH 5.2) as loading buffer and run at 120 V for 2.25 h for the gel with pTyr. The gel with pSer and pThr was run at 200 V for 1 h followed by 250 V for 30 min. After the run the gel was washed with MilliQ and FAM fluorescence was visualized on the Bio-Rad gel scanner and roughly quantified by ImageJ. (see Supplementary data for the complete gels)

### Phosphate stability

In a 0.6 mL Eppendorf tube were added 7.5 µL $H_2O$, 1.5 µL HEPES-KOH (500 mM, pH 7.5), 3 µL $MgCl_2$ (3 M), and 3 µL pSer (25 mM). The mixture was incubated on ice and in the dark. Samples were taken at the start, after 5 hours, and after 3 days. Samples were analysed by LC-MS with a 5-95% buffer B gradient over 10 min.

MS(ESI-API) (m/z): Calc. for $C_{10}H_{13}N_3O_{10}P$ $[M+H]^+$366.03; Found 366.10

## Translation of GFP and TSC2_979-988

### Aminoacylation of tRNA

In a tube were added $H_2O$, HEPES-KOH (pH 7.5, 50 mM), dFx (25 µM), and the desired tRNA (25 µM). The reaction mixture was heated for 2 min at 95 °C, followed by 5 min at rt. $MgCl_2$ (300 mM) was added and the mixture was again left to rest for 5 min at rt. The mixture was chilled on ice before adding the amino acid (2.5 mM), and the complete mixture was incubated on ice in the dark overnight (approximately 17 hours).

Afterwards, the product was precipitated by acetic acid and ethanol. The mixture was first diluted 5 times with 0.3 M AcOH and afterwards 3 times more diluted with 100% ethanol. The mixture was vortexed and centrifuged at 15 000 x g for 15 min to form a pellet, and the supernatant was discarded. The pellet was resuspended in 70% ethanol and 0.1 M AcOH in $H_2O$. The suspension was vortexed and centrifuged at 15 000 x g for 10 min to form a pellet, and the supernatant was again discarded. Resuspending the pellet and centrifuging were executed again. Afterwards, the pellet was washed by adding 70% ethanol, centrifuging at 15 000 x g for 3 min, discarding the supernatant. The created acylated tRNA pellets were dried at rt and then stored at -20 °C until usage.

### DNA template preparation

The DNA template for GFP Wt (pET28a sfGFP) and GFP-TAG (pET28a sfGFP 150TAG) (35) were a kind gift from dr. Drienovska (VU Amsterdam), amplified in Escherichia coli DH5$\alpha$ and purified using a peqGOLD Plasmid Miniprep Kit I (VWR International B.V., NL). The DNA templates for TSC2 were synthesized by primer extension in PCR. Three DNA templates were created: TSC2-TAG, TSC2-TGA, and TSC2-AGC. (see Supplementary data for sequences)

Forward primer T7g10M.F48 was used in combination with one of the following reverse primers: TSC2-TAG1.R60 for TSC2-TAG, TSC2-TGA1.R60 for TSC2-TGA, or TSC2-AGC1.R60 for TSC2-AGC. The primers were extended by *KOD* polymerase in a mixture consisting of *KOD* buffer (1x), dNTPs (250 µM each), $MgCl_2$ (1 mM), *KOD* working enzyme (1x), primers (0.25 µM each), and $H_2O$ in a final volume of 20 µL. PCR was performed by first melting at 95 °C for 1 min, followed by 5 cycles of 55 °C for 1 min and 72 °C for 1 min, and ending in 72 °C for 5 min to complete final extensions. (see Supplementary data for primer sequences)

For the final extensions, the primers T7g10M.F48 and TSC-2.R58 were used for all three templates. The reaction mixture consisted of the same components was the first extension but another primer concentration (2.5 µM) and extension mixture (1%). PCR was performed by first melting at 95°C for 1 min, followed by 12 cycles of 95°C for 40 sec, 61 °C for 40 sec, and 72 °C for 40 sec, and ending in 72 °C for 5 min to complete extensions. 2 µL of each mixture was analysed on 3% agarose gel run at 250 V for 8 min in SB buffer with SyBR Safe DNA gel stain. (see Supplementary data for the gel)

The DNA template was then purified by ethanol precipitation and the pellets were dissolved in RNase free $H_2O$ and adjusted to a final concentration of 1 mM.

## Translation

### *GFP translation*

For all translations of GFP the PURExpress® Δ RF123 Kit (NEB Inc., USA) was used. The translations were performed by mixing the components as mentioned in Table 1 Table 1. Translation reaction mixtures per sampleand incubated for 2h. The fluorescence was visualized by scanning the Eppendorf tubes in an Azure C200 gel scanner (EPI Blue light source, 100 ms exposure). The tubes were stored at -20 °C before quantification by plate reader. The translations were diluted with 45 µL 0.01 M PBS (pH 8.0) and transferred to a Greiner 96-well white flat bottom plate. The plate was analysed with 470-426 excitation, 515-520 emission, and a gain of 750.

*Table 1. Translation reaction mixtures per sample*

| *Reagent* | *GFP Wt* | *No DNA template* | *Negative* | *Reprogrammed GFP* |
|---|---|---|---|---|
| Sol A | 2 µL | 2 µL | 2 µL | 2 µL |
| Sol B ΔRF | 1.5 µL | 1.5 µL | 1.5 µL | 1.5 µL |
| RF1 | 0.1 µL | | | |
| RF2 | 0.1 µL | 0.1 µL | 0.1 µL | 0.1 µL |
| RF3 | 0.1 µL | 0.1 µL | 0.1 µL | 0.1 µL |
| RNase inhibitor | 0.2 µL | 0.2 µL | 0.2 µL | 0.2 µL |
| Pellet (max. 3 µg in 1mM AcOH) | | | | 0.5 µL |
| $H_2O$ | 0.6 µL | 1.1 µL | 0.7 µL | 0.2 µL |
| DNA template | 0.4 µL | | 0.4 µL | 0.4 µL |
| *Total* | *5 µL* | *5 µL* | *5 µL* | *5 µL* |

### *ZipTip purification*

A ZipTip C18 Pipette Tip (Merck Millipore Ltd., IE) was first washed with 15µL 80% and 15 µL 4% ACN in $H_2O$. The translation mixture was then loaded on the tip, washed with 15 µL 4% ACN in $H_2O$, and the product was eluted with 10 µL 80% ACN in $H_2O$. The obtained elution was then diluted with 5 µL $H_2O$ for further steps.

### *TSC2-TAG translation*

For all translations of TSC2 with TAG reprogramming, the PURExpress® Δ RF123 Kit from NEB Inc. was used. For a 50 µL scale translation were mixed following manufacturer's protocol with pSer-EnGlu pellet (max. 18 µg) in 7.75 µL $H_2O$, and 5 µL TSC-TAG template (1 mM). The mixture was prepared on ice and afterwards incubated at 37 °C for 30 min. 20 µL of the reaction mixture was digested with 10 µg trypsin and analysed on an orbitrap-QE by the Altelaar group. (see Supplementary data for more details)

For direct LC-MS analysis, the same reaction was performed again but scaled down to 5 µL total. The reaction mixture was purified by ZipTip. The obtained elution was injected on LC-MS with a 5-95% buffer B gradient over 22 min.

MS(ESI-API) (m/z): Calc. for $C_{109}H_{169}N_{31}Na_2O_{31}S$ $[M+2Na+4H]^{4+}$ 621.6; Found 621.6

*Natural translation TSC2-AGC*

For the natural translation of the TSC2-AGC template, components of the PURExpress® Δ RF123 Kit and PURExpress® Δ (aa, tRNA) Kit from NEB Inc. were used. To a 0.6 mL low-binding Eppendorf tube were added 1.5 µL Sol B (from the Δ(aa, tRNA) Kit), 2 µL Sol A (from the ΔRF123 Kit), 0.2 µL RNase inhibitor (murine), 0.8 µL $H_2O$, and 0.5 µL TSC2-AGC template (1 mM). The reaction was incubated at 37 °C for 30 minutes. The obtained mixture was then purified by ZipTip, and the obtained elution was injected on LC-MS with a 5-95% buffer B gradient over 22 min.

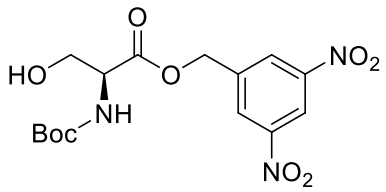
MS(ESI-API) (m/z): Calc. for $C_{109}H_{170}N_{31}O_{31}S$ $[M+3H]^{3+}$ 813.7 Found 814.5

*AGC reprogramming*

For all translations of TSC2 with AGC reprogramming, components of the PURExpress® Δ RF123 Kit and PURExpress® Δ (aa, tRNA) Kit from NEB Inc. were used. To 2 µL Sol A (ΔRF123 Kit) was added 0.5 µL M5-SerGCU-suppressor (100 µM). The mixture was incubated at 37 °C for 5 min and chilled on ice afterwards. The acylated tRNA pellet (max. 3 µg) was dissolved in 0.3 µL $H_2O$ and added to the mixture. This mixture was then added to 1.5 µL Sol B (Δ(aa, tRNA) Kit), 0.2 µL RNase inhibitor (murine), and 0.5 µL TSC2-AGC template (1 mM). The reaction was incubated at 37 °C for 30 minutes. The obtained mixture was then purified by ZipTip, and the obtained elution was injected on LC-MS with a 5-95% buffer B gradient over 22 min. No expected products were found.

# Syntheses

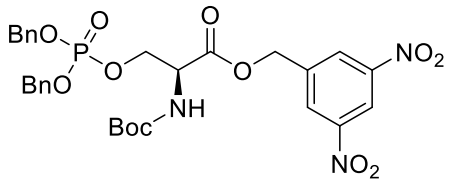
## Boc-Ser-DBE **(2)**



Boc-Ser-OH (634 mg, 3.09 mmol), DNB-Cl (735.3 mg, 3.40 mmol), and $Et_3N$ (1.0 mL, 7.2) were added to DMF (5 mL) and stirred in the dark and heated to 50 °C for 24h. The crude product was purified with flash column chromatography (EA:PE 1:2 then 1% → 2.5% MeOH in DCM) to obtain fluffy white solid as Boc-Ser-DBE (670.4 mg, 1.74 mmol, 56%). NMR data were consistent with those previously reported (11).

$^1$H NMR (400 MHz, $CDCl_3$) δ 8.99 (t, *J* = 2.1 Hz, 1H), 8.58 (d, *J* = 1.9 Hz, 2H), 5.52 – 5.31 (m, 3H), 4.48 (s, 1H), 4.14 – 4.06 (m, 1H), 3.96 (ddd, *J* = 10.9, 5.0, 3.4 Hz, 1H), 1.44 (s, 12H).

## Boc-Ser(PO(OBn)$_2$)-DBE **(3)**

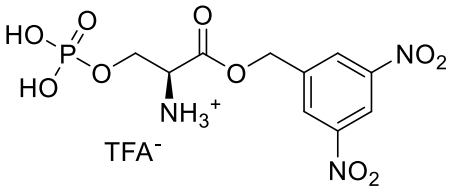


A solution of Boc-Ser-DBE **(2)**(192.4 mg, 0.50 mmol) and dibenzyl diisopropylphosphoramidite (0.25 mL, 0.74 mmol) was coevaporated with toluene (3x 10 mL) and dissolved in DCM (3 mL) and ACN (0.9 mL). A solution of 1H-tetrazole in ACN (0.45 M, 2.1 mL, 0.95 mmol) was added and the reaction was stirred with Ar atmosphere at room temperature. The reaction was monitored with TLC (EA:PE 1:1, Rf = 0.79) and was completed after 35 min.

To the reaction mixture, hydrogen peroxide (30% w/v, 0.38 mL, 3.4 mmol) was added at 0 °C and stirred for another 25 min. The reaction mixture was then diluted with DCM, washed with $H_2O$, dried over $Na_2SO_4$ and solvent was then removed by rotary evaporation. The crude product was further purified by flash column chromatography (EA:Toluene 1:5 → 1:1) to give Boc-Ser(PO(OBn)$_2$)-DBE (304.9 mg, 0.47 mmol, 94.6%).

$^1$H NMR (500 MHz, $CDCl_3$) δ 8.96 (t, *J* = 2.1 Hz, 1H), 8.53 (d, *J* = 2.1 Hz, 2H), 7.43 – 7.31 (m, 10H), 5.52 (d, *J* = 8.5 Hz, 1H), 5.28 (s, 2H), 5.09 – 4.96 (m, 4H), 4.49 (ddd, *J* = 11.5, 8.7, 3.2 Hz, 1H), 4.22 (ddd, *J* = 10.2, 6.9, 2.9 Hz, 1H), 1.48 (s, 9H).

## H-Ser(P)-DBE **(4)**



To DCM (15 mL) was added Boc-Ser($PO(OBn)_2$)-DBE **(3)** (69.9 mg, 0.108 mmol) and TFA (5 mL) to give a yellow solution. The reaction was monitored with TLC (EA:MeOH:$H_2O$ 7:2:1, ninhydrin stain) and was completed after 18 h. The solvent and TFA was removed by rotary evaporation and covap with toluene (3x). The crude product was dissolved in DMSO and further purified by preparative HPLC with a 5-50% buffer B gradient for 50 min at 12.5 mL/min. The obtained fractions with product were grouped and lyophilized to give H-Ser(P)-DBE as TFA salt (75.5 mg, 0.157 mmol). The yield could not be determined as the product was crystallized with an unknown amount of $H_2O$ and TFA.

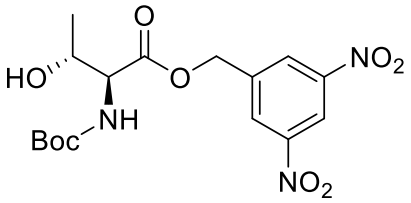
$^1$H NMR (500 MHz, DMSO) δ 8.81 (t, *J* = 2.1 Hz, 1H), 8.75 (d, *J* = 2.1 Hz, 2H), 5.59 – 5.43 (m, 2H), 4.48 (t, *J* = 3.5 Hz, 1H), 4.23 (dd, *J* = 8.0, 3.7 Hz, 2H).

$^{13}$C NMR (126 MHz, DMSO) δ 166.87, 148.12, 139.40, 128.75, 118.48, 65.33, 63.19, 52.76.

$^{31}$P NMR (202 MHz, DMSO) δ -0.95.

MS(ESI-API) (m/z): Calc. for $C_{10}H_{13}N_3O_{10}P$ $[M+H]^{1+}$366.03; Found 366.10

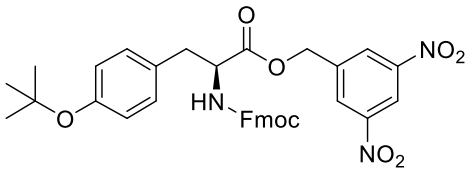
Boc-Thr-DBE (12)




Boc-Thr-OH (830.1 mg, 3.79 mmol), DNB-Cl (912.3 mg, 4.21 mmol), and $Et_3N$ (0.8 mL, 5.73) were added to DMF (10 mL) and stirred in the dark for 4 days at room temperature. The product was extracted with DCM instead of $Et_2O$ and flash chromatography was performed with EA:PE (1:3 → 1:1) as eluent to give Boc-Thr-DBE (1.27 mg, 3.17 mmol, 84%). NMR data were consistent with those previously reported (11).

$^1$H NMR (500 MHz, $CDCl_3$) δ 9.00 (t, *J* = 2.1 Hz, 1H), 8.58 (d, *J* = 2.0 Hz, 2H), 5.46 – 5.36 (m, 2H), 5.33 (d, *J* = 9.2 Hz, 1H), 4.44 (dt, *J* = 7.9, 3.9 Hz, 1H), 4.40 – 4.34 (m, 1H), 1.45 (s, 9H), 1.31 (d, *J* = 6.4 Hz, 3H).

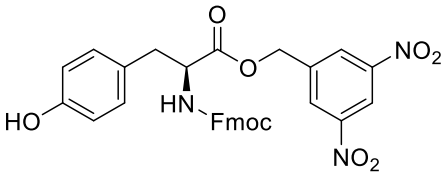
Fmoc-Tyr(tBu)-DBE (16)




To DMF (5 mL) was added Fmoc-Tyr(tBu)-OH (1.2221 g, 2.66 mmol), DNB-Cl (0.4985 g, 2.30 mmol), and DIPEA (0.6 mL, 3.44 mmol). The mixture was stirred at rt as a yellow liquid that later changed to brown. The reaction was monitored by TLC (EA:PE 1:2) and after 24 h EA (250 mL) was added. The organic layer was washed with brine (150 mL), 1 M $KHSO_4$ (150 mL), sat. aq. $NaHCO_3$ (150 mL) (2x), and brine (150 mL). The organic layer was dried over $Na_2SO_4$, and the solvents were removed *in vacuo* to yield a yellow oil. The crude oil was further purified by flash column chromatography (EA:PE 1:3 → 1:1). Solvent was removed *in vacuo* to yield Fmoc-Tyr(tBu)-DBE as a yellow oil (1.22 g, 1.91 mmol, 83%). NMR data were consistent with those previously reported (36).

$^{1}$H NMR (400 MHz, $CDCl_3$) δ 8.98 – 8.89 (m, 1H), 8.39 (s, 2H), 7.73 (dq, *J* = 7.6, 1.1 Hz, 2H), 7.54 (d, *J* = 7.5 Hz, 2H), 7.38 (ddt, *J* = 9.6, 7.5, 2.0 Hz, 2H), 7.28 (tt, *J* = 8.7, 1.7 Hz, 2H), 6.96 (d, *J* = 8.1 Hz, 2H), 6.79 (d, *J* = 8.0 Hz, 2H), 5.33 (s, 1H), 5.20 (s, 2H), 4.66 (q, *J* = 7.1 Hz, 1H), 4.39 (t, *J* = 5.7 Hz, 2H), 4.22 – 4.13 (m, 1H), 3.06 (qd, *J* = 13.8, 6.8 Hz, 2H), 1.28 (s, 11H).

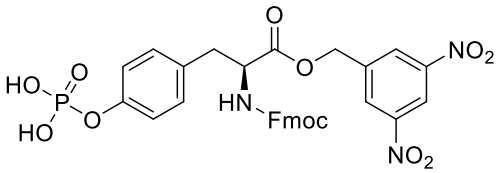
Fmoc-Tyr-DBE **(17)**




To DCM (25 mL) was added Fmoc-Tyr(tBu)-DBE **(16)** (1.22 g, 1.91 mmol) and TFA (25 mL). The reaction was monitored by TLC (EA:PE 1:2) and after 2 h the solution was filtered, and solvent was removed *in vacuo* and by covap with toluene (3x). The product was dried on the high vac to yield Fmoc-Tyr-DBE as a yellow crystal (890 mg, 1.53 mmol, 80%).

$^{1}$H NMR (400 MHz, $CDCl_3$) δ 8.90 (t, *J* = 2.2 Hz, 1H), 8.32 (d, *J* = 2.0 Hz, 2H), 7.73 (d, *J* = 7.5 Hz, 2H), 7.53 (d, *J* = 7.5 Hz, 2H), 7.41 – 7.26 (m, 4H), 6.91 (d, *J* = 8.0 Hz, 2H), 6.64 (d, *J* = 8.0 Hz, 2H), 5.31 (d, *J* = 8.0 Hz, 1H), 5.17 (s, 2H), 4.64 (q, *J* = 7.2 Hz, 1H), 4.47 – 4.34 (m, 2H), 4.16 (t, *J* = 6.9 Hz, 1H), 3.11 – 2.92 (m, 2H).

Fmoc-Tyr(P)-DBE **(18)**

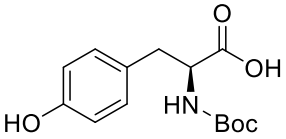



To $Et_2O$ (1 mL) was added $POCl_3$ (0.03 mL, 0.322 mmol) and $Et_3N$ (0.13 mL, 0.933 mmol) and white precipitation and fume formed. Fmoc-Tyr-DBE **(17)** (102.0 mg, 0.159 mmol) was dissolved in $Et_2O$ (1.5 mL) and DCM (3 mL) and added dropwise to the $POCl_3$ mixture which formed a white fume and orange opaque solution. The reaction was monitored by TLC (EA:PE 1:1) and $H_2O$ (10 mL) was added after 1 h. The organic layer was extracted, and the remaining aqueous layer was extracted more by EA (2x 30 mL). The combined organic layers were dried over $Na_2SO_4$, and the solvent was removed *in vacuo*. The product was further dried on the high vac to yield Fmoc-Tyr(P)-DBE (122.9 mg, 0.185 mmol). The yield could not be determined as the product still contained product with chlorides instead of hydroxyls on the phosphate group.

$^{1}$H NMR (500 MHz, $CDCl_3$) δ 8.84 (s, 1H), 8.35 (s, 2H), 7.84 – 6.88 (m, 16H), 5.17 (d, *J* = 38.7 Hz, 3H), 4.34 (s, 2H), 4.10 (s, 2H), 3.07 (s, 4H).

$^{31}$P NMR (202 MHz, $CDCl_3$) δ -1.73.

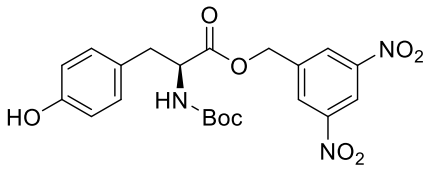
Boc-Tyr-OH **(23)**



To a suspension of L-Tyrosine (506.7 mg, 2.8 mmol) in $H_2O$ (25 mL) was added $NaHCO_3$ (359.0 mg, 4.3 mmol) and $Boc_2O$ (905.2 mg, 4.1 mmol) in MeOH (30 mL). The reaction was stirred at room temperature and monitored by TLC (EA:MeOH:$H_2O$ 7:2:1, Rf = 0.66). After 2 days, more $Boc_2O$ (399.8 mg, 1.83 mmol) was added since not all L-Tyrosine (Rf = 0.24) had reacted. Reaction was stopped after a total of 3 days stirring. MeOH was removed by rotary evaporation and the aqueous solution was acidified with 1M HCl. The product was extracted with EA (3x 50 mL), dried over $Na_2SO_4$, and rotary evaporated to yield Boc-Tyr-OH as white crystal (652.2 mg, 2.32 mmol, 83%).

NMR data were consistent with those previously reported (37).

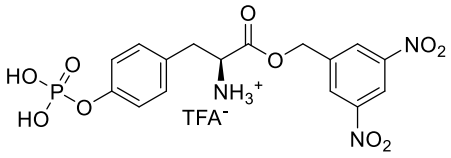
## Boc-Tyr-DBE (24)




To a solution of Boc-Tyr-OH **(23)** (652.2 mg, 2.3 mmol) in DMF (5 mL) was added DNB-Cl (371.1 mg, 1.7 mmol) and $Et_3N$ (0.5 mL, 3.6 mmol). The yellow solution was stirred at room temperature in the dark for 24h to obtain a purple solution. TLC showed formation of product (EA:PE 1:2). The product was extracted with $Et_2O$, and washed with 1 M HCl, sat. $NaHCO_3$, and brine. The aqueous layers were once more extracted with DCM. Organic layers were dried over $Na_2SO_4$, and solvent was evaporated. The crude product was further purified by flash column chromatography (EA:PE 1:3) to obtain Boc-Tyr-DBE as a yellow solid (584.2 mg, 1.27 mmol, 74%). NMR data were consistent with those previously reported (11).

$^1$H NMR (500 MHz, $CDCl_3$) δ 9.00 (s, 1H), 8.38 (d, *J* = 2.0 Hz, 2H), 6.97 (d, *J* = 8.4 Hz, 2H), 6.71 – 6.62 (m, 2H), 5.23 (s, 2H), 4.98 (d, *J* = 7.4 Hz, 1H), 4.82 (s, 1H), 4.57 (dd, *J* = 14.1, 7.2 Hz, 1H), 3.00 (ddd, *J* = 50.2, 13.8, 6.9 Hz, 2H), 1.42 (s, 8H).

## H-Tyr(P)-DBE (25)




In a round bottom flask with $Et_2O$ (10 mL) was added $POCl_3$ (0.1 mL, 1.1 mmol) and $Et_3N$ (0.5 mL, 3.6 mmol), white precipitate formed immediately. Boc-Tyr-DBE **(24)** (392.3 mg, 0.850 mmol) in $Et_2O$ (30 mL) was added dropwise. The reaction was stirred at room temperature and a pH of approximately 9 was observed. The reaction was monitored by TLC (EA:PE 1:1, Rf = 0.0) and all starting material had reacted after 30 min.

To the reaction flask, $H_2O$ (30 mL) was added and stirred for another 30 min. White fume formed while adding $H_2O$. The product was extracted by EA and dried over $Na_2SO_4$. The solvent was removed by rotary evaporation. The product was further purified by flash column chromatography (DCM:MeOH 9:1). However, product was only observed after flushing the column with MeOH. Boc-Tyr(P)-DBE was obtained in a crude yellow oil.

To half of the MeOH flush with Boc-Tyr(P)-DBE was added TFA (3 mL) and stirred for 30 min. The solvent and TFA were partially removed *in vacuo*, and the product was precipitated in cold $Et_2O$ (30 mL) and centrifuged at 4500 rpm for 5 min. Washing with $Et_2O$ (30 mL) and centrifuging was repeated 3 times. The precipitate was afterwards dried on air at room temperature, redissolved in 20% ACN in $H_2O$ (5 mL), and first 1 mL was injected for preparative HPLC to further purify the product. The fractions were collected with a gradient of 5-70% buffer B for 110 min at 12.5 mL/min. Afterwards, the remainder was purified with a gradient of 5-40% buffer B for 50 min at 12.5 mL/min. The obtained fractions with product were lyophilized to yield H-Tyr(P)-DBE as a white solid (251.8 mg, 0.57 mmol). The yield could not be determined as the product was crystallized with an unknown amount of $H_2O$ and TFA.

$^1$H NMR (500 MHz, DMSO) δ 8.82 (d, *J* = 2.2 Hz, 1H), 8.67 (d, *J* = 2.1 Hz, 2H), 7.18 (d, *J* = 8.2 Hz, 2H), 7.05 (d, *J* = 8.1 Hz, 2H), 5.51 – 5.39 (m, 2H), 4.44 (t, *J* = 6.7 Hz, 1H), 3.10 (d, *J* = 6.7 Hz, 2H).

$^{13}$C NMR (126 MHz, DMSO) δ 168.77, 148.04, 139.25, 130.37, 128.60, 120.08 (d, *J* = 4.9 Hz), 118.54, 65.02, 53.22, 35.26.

$^{31}$P NMR (202 MHz, DMSO) δ -5.92.

MS(ESI-API) (m/z): Calc. for $C_{16}H_{17}N_3O_{10}P$ $[M+H]^{1+}$442.06; Found 442.10
MS(ESI-API) (m/z): Calc. for $C_{16}H_{15}N_3O_{10}P$ $[M-H]^{1-}$440.05; Found 440.00

## Strep-tag synthesis

H—W—S—H—P—Q—F—E—K—OH

The Strep-tag was synthesized by automated SPPS on a Liberty Blue (CEM). The deprotectant was 20% piperazine in DMF, the activator was 0.25 M DIC in DMF, and the base used was 0.25 M Oxyma in DMF. The amino acid solutions were all concentrations of 0.2 M. Cl-MPA ProTide (LL) resin (628 mg, 1 mmol) in DCM:DMF (1:1, 10 mL) was preloaded with L-lysine. Concentration of preloaded peptide was then determined by cleavage of the Fmoc group as follows. To dry preloaded resin (30.5 mg) was added 20% piperidine (10 mL) and left shaking for 20 min. The solution was centrifuged, and 0.1 mL of the supernatant was diluted with 0.9 mL MeOH. The supernatant was analysed with UV-VIS (300 nm, 1 cm path length, extinction coefficient: 6000 $M^{-1}cm^{-1}$ and a yield of 84% was determined. The Strep-tag was synthesized on the above preloaded resin (0.188 g, 0.025 mmol) and afterwards washed with DCM. The peptide was cleaved and deprotected in TFA (2.78 mL), $H_2O$ (150 µL), and TIPS (75 µL) for 1 h. Afterwards, it was precipitated from cold $Et_2O$ (30 mL) and centrifuged at 4500 rpm for 5 min. Washing of the pellet with $Et_2O$ (30 mL) and centrifuging was repeated 3 times. The precipitate was dissolved in 1 mL DMSO, and the product purified by preparative HPLC with 5-30% buffer B for 50 min at 12.5 mL/min. The obtained fractions confirmed to contain product were lyophilized to yield Strep-tag as a white solid.

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(Figures were made on Biorender.com)

# Supplementary data

Primers & DNA templates

| *Primer* | *Sequence* |
|---|---|
| *T7g10M.F48* | TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATG |
| *TSC2-AGC1.R60* | CCAGCGCACCACATGTTCGGACACGCTAATGGACTTCATATGTATATCTCCTTCTTAAAG |
| *TSC2-TAG1.R60* | CCAGCGCACCACATGTTCGCTCACCTAAATGCTCTTCATATGTATATCTCCTTCTTAAAG |
| *TSC2-TGA1.R60* | CCAGCGCACCACATGTTCGCTCACTCAAATGCTCTTCATATGTATATCTCCTTCTTAAAG |
| *TSC-2.R58* | CCGCTGAGCAATAACTAGCTTACTTTTCAAACTGCGGATGGGACCAGCGCACCACATG |

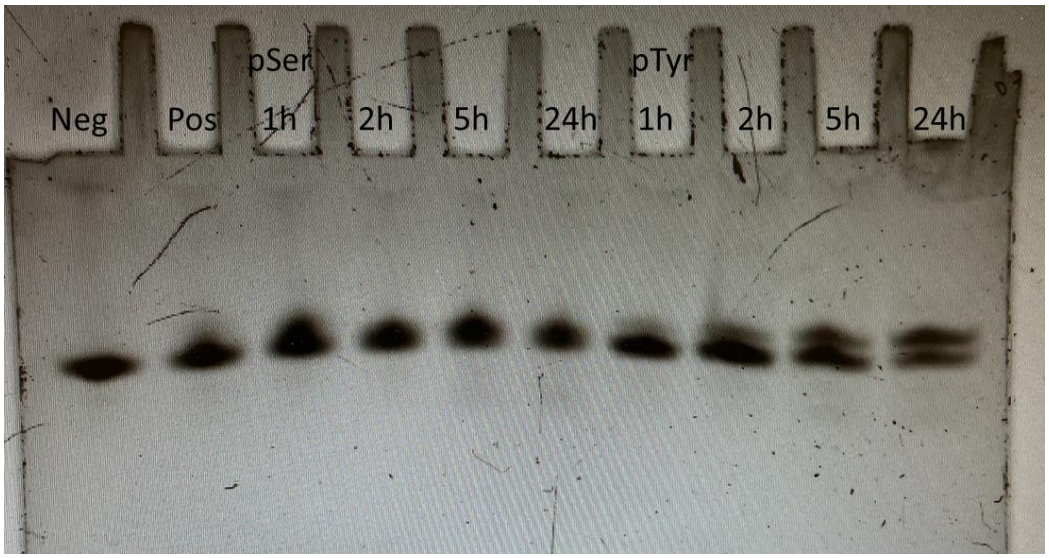
| *DNA template* | *Sequence* |
|---|---|
| *TSC2-AGC* | TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACAT ATG AAG TCC ATT AGC GTG TCC GAA CAT GTG GTG CGC TGG TCC CAT CCG CAG TTT GAA AAG TAAGCTAGTTATTGCTCAGCGG |
| *TSC2-TAG* | TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACAT ATG AAG AGC ATT TAG GTG AGC GAA CAT GTG GTG CGC TGG TCC CAT CCG CAG TTT GAA AAG TAAGCTAGTTATTGCTCAGCGG |
| *TSC2-TGA* | TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACAT ATG AAG AGC ATT TGA GTG AGC GAA CAT GTG GTG CGC TGG TCC CAT CCG CAG TTT GAA AAG TAAGCTAGTTATTGCTCAGCGG |
| *Peptide* | *MKSI**S**VSEHVVRWSHPQFEK** |

**possible vacant codon in bold and underlined*

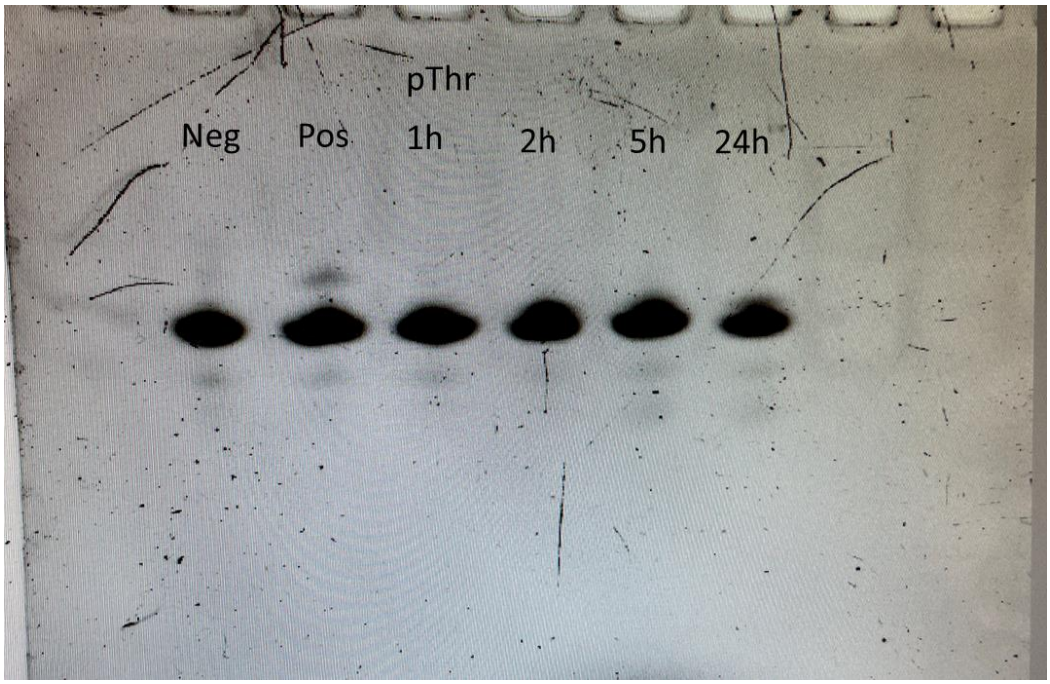
Gel images

*Aminoacylation efficiency test*

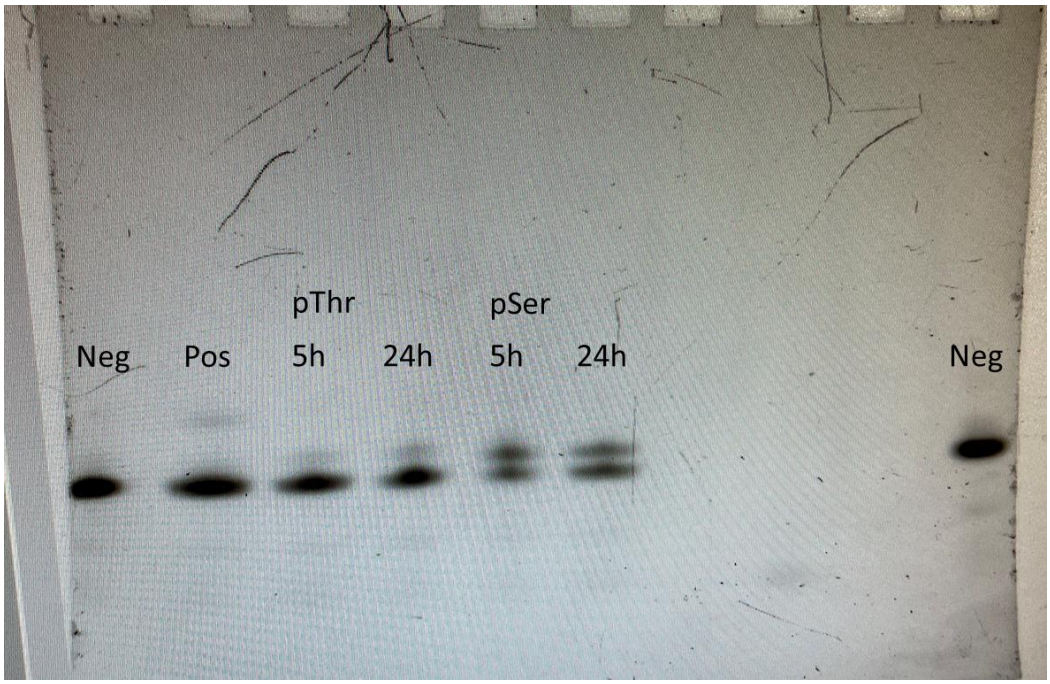
First gel, pSer and pTyr:



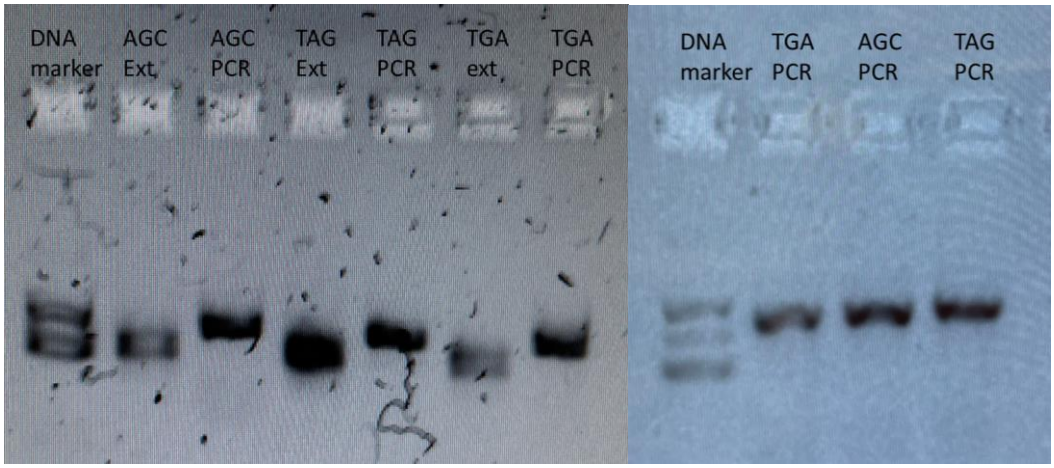
Second gel, pThr:




Third gel, pThr and pSer:



DNA template – PCR amplication




LC-MS summary

| *Sample* | *Signal* | *Rt (min)* | *Calculated m/z* | *Found m/z* |
|---|---|---|---|---|
| $Mg^{2+}$ stability – pSer | Positive | 6.147 | $[M+H]^{1+}$ 366.03 | 366.10, 731.10 |
| | Negative | 6.143 | $[M-H]^{1-}$ 364.02 | 729.0 |
| TSC-TAG translation – Ser | Positive | 9.163 | $[M+2Na+4H]^{4+}$ 621.5525 | 621.6, 497.4, 828.5 |
| TSC-AGC natural translation | Positive | 9.077 | $[M+3H]^{3+}$ 813.7467 | 814.5, 489.1, 611.0 |
| H-Ser(P)-DBE (4) | Positive | 6.019 | $[M+H]^{1+}$ 366.03 | 366.10, 731.10 |
| H-Tyr(P)-DBE (25) | Positive | 6.470 | $[M+H]^{1+}$ 442.06 | 442.10, 883.10 |
| | Negative | 6.472 | $[M-H]^{1-}$ 440.05 | 440.00, 881.10 |
| H-Thr(P)-DBE – DNB-OH | Negative | 1.613 | $[M-H]^{1-}$ 197.02 | 198.10, 397.00 |
| H-Thr(P)-DBE (14) | Positive | 6.219 | $[M+H]^{1+}$ 380.05 | 380.10, 759.20 |
| | Negative | 6.215 | $[M-H]^{1-}$ 378.03 | 757.10 |

Orbitrap-QE method

LC gradient: buffer A ($H_2O$, 0.1% TFA), buffer B (80% ACN, 0.1% TFA), 13-44% buffer B for 60 min.

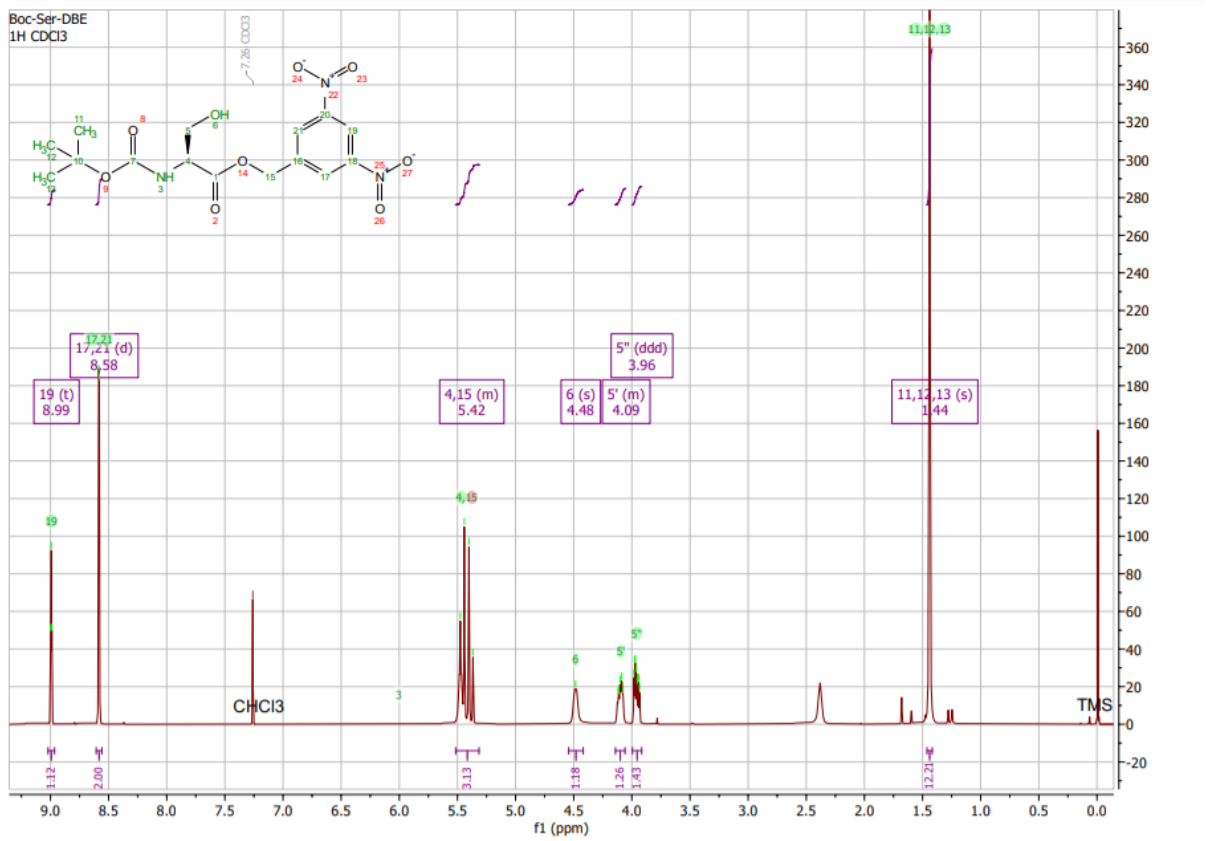
MS1: resolution 60 000, AGC target 3e6, max IT: 120 msec, scan range: 375-1600 m/z, topN = 10

MS2: Resolution 30 000, AGC target 1e5, max IT: 120 msec, NCE: 27

NMR and HPLC spectra

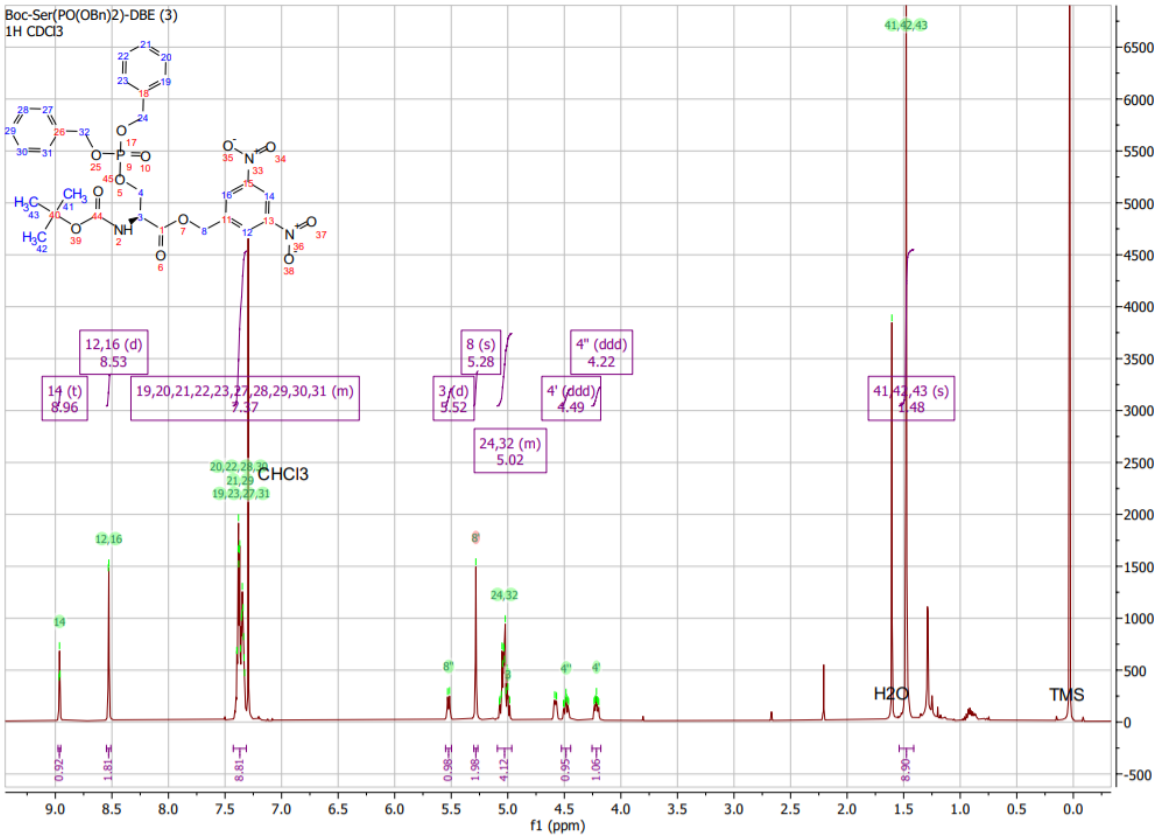
*Boc-Ser-DBE* ***(2)***

$^{1}$H NMR

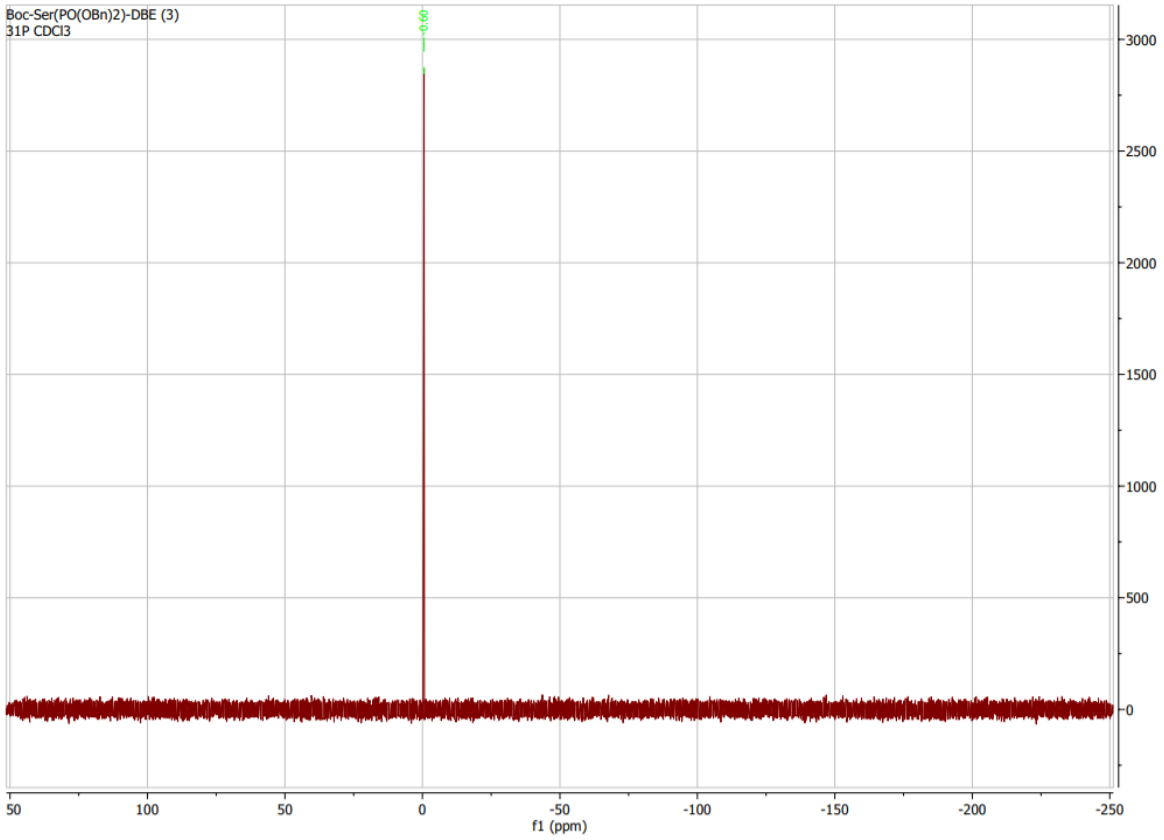


*Boc-Ser(PO(OBn)$_2$)-DBE* ***(3)***

$^1$H NMR

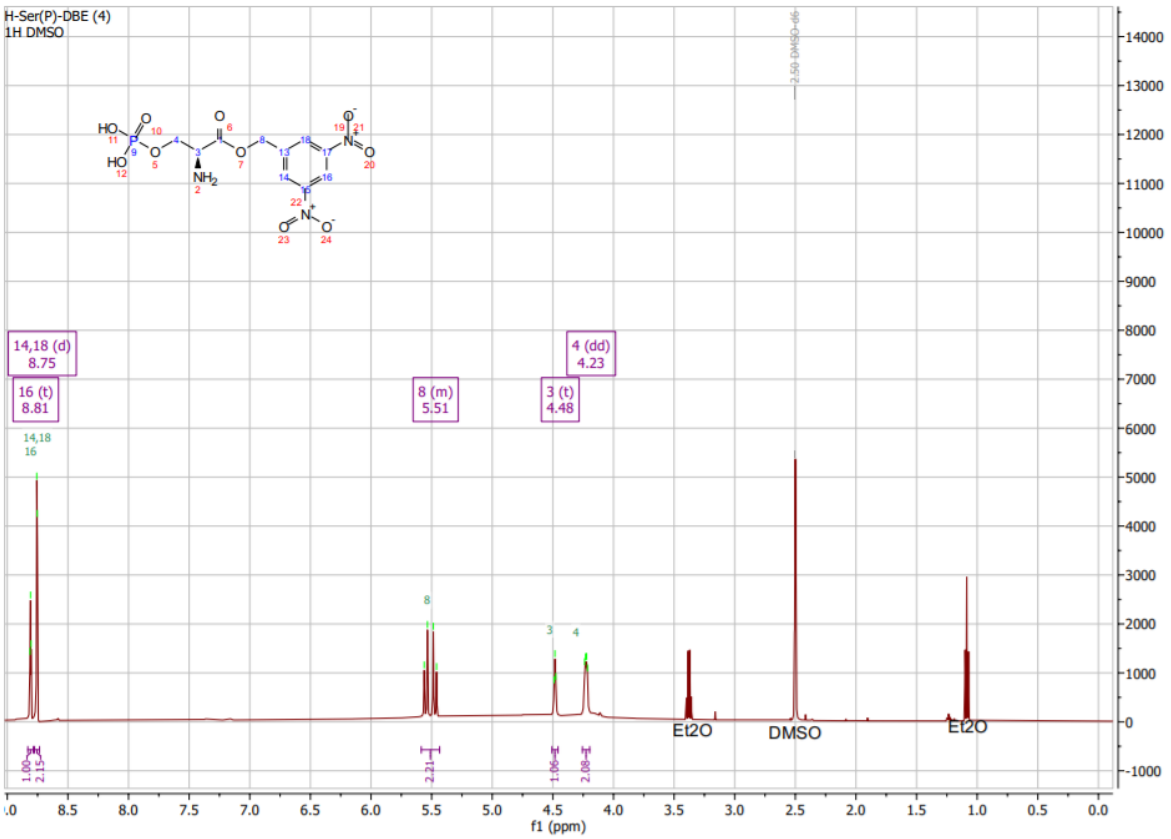



$^{31}$P NMR

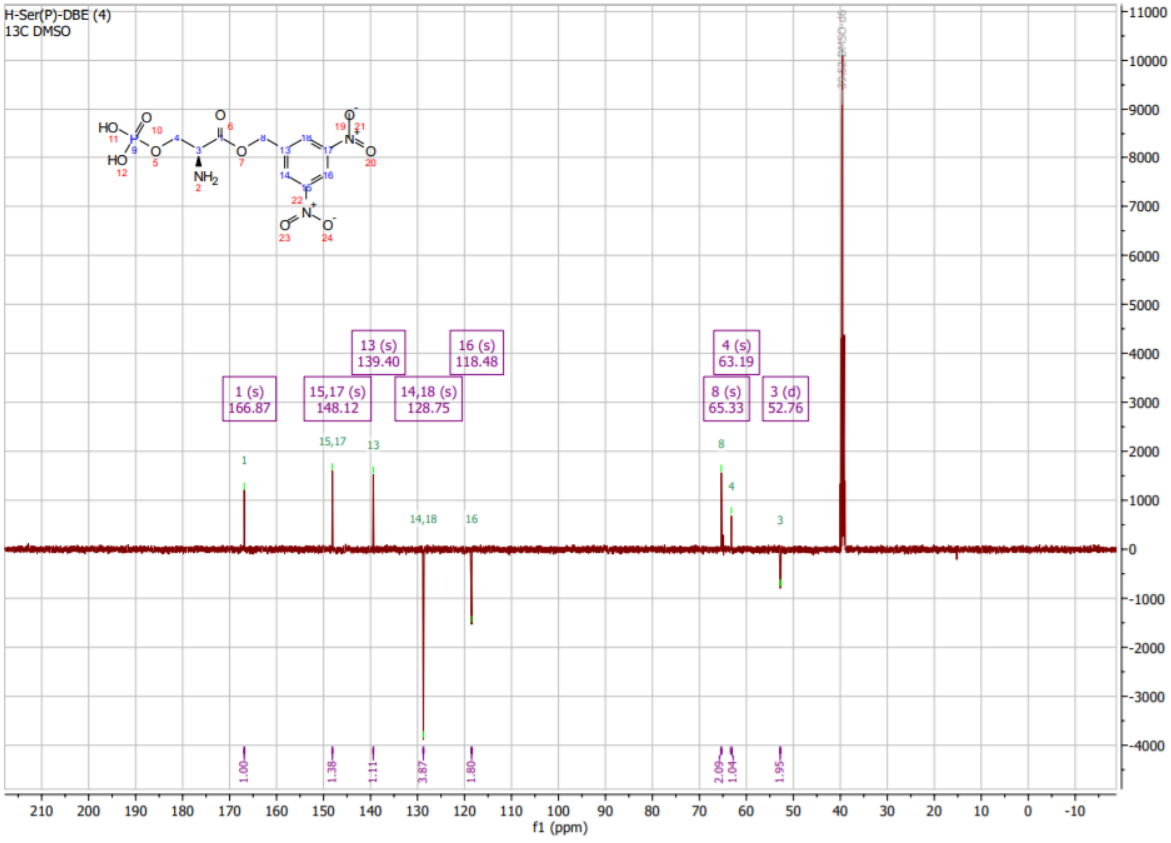


*H-Ser(P)-DBE* ***(4)***

$^{1}$H NMR




$^{13}$C NMR



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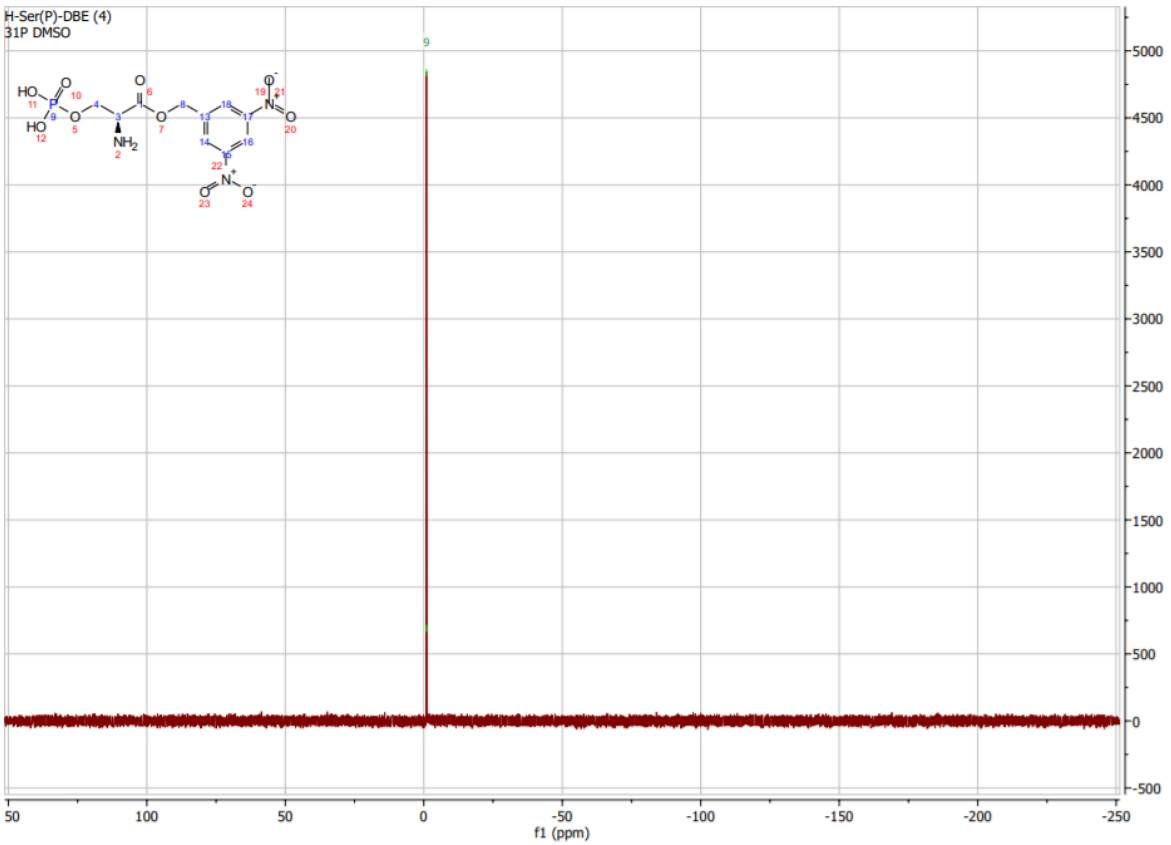
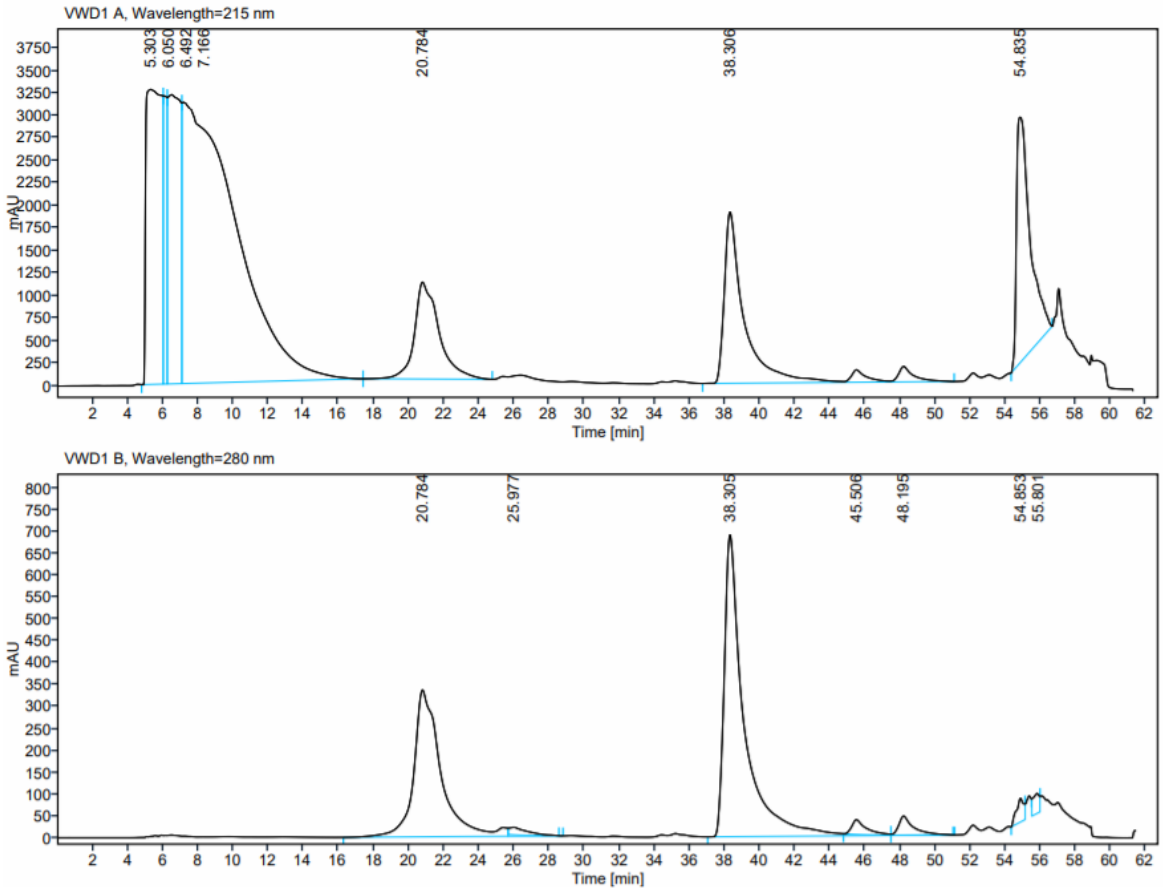
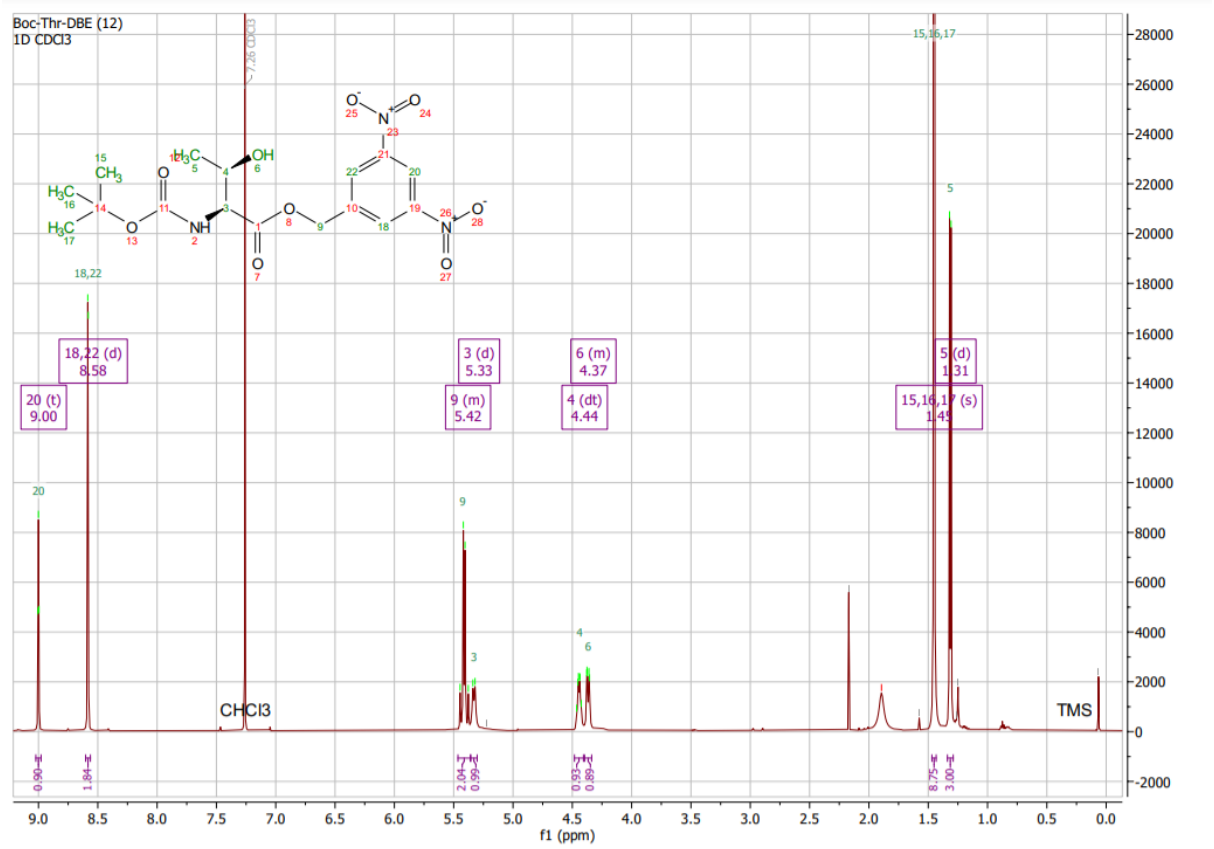



Preparative HPLC traces



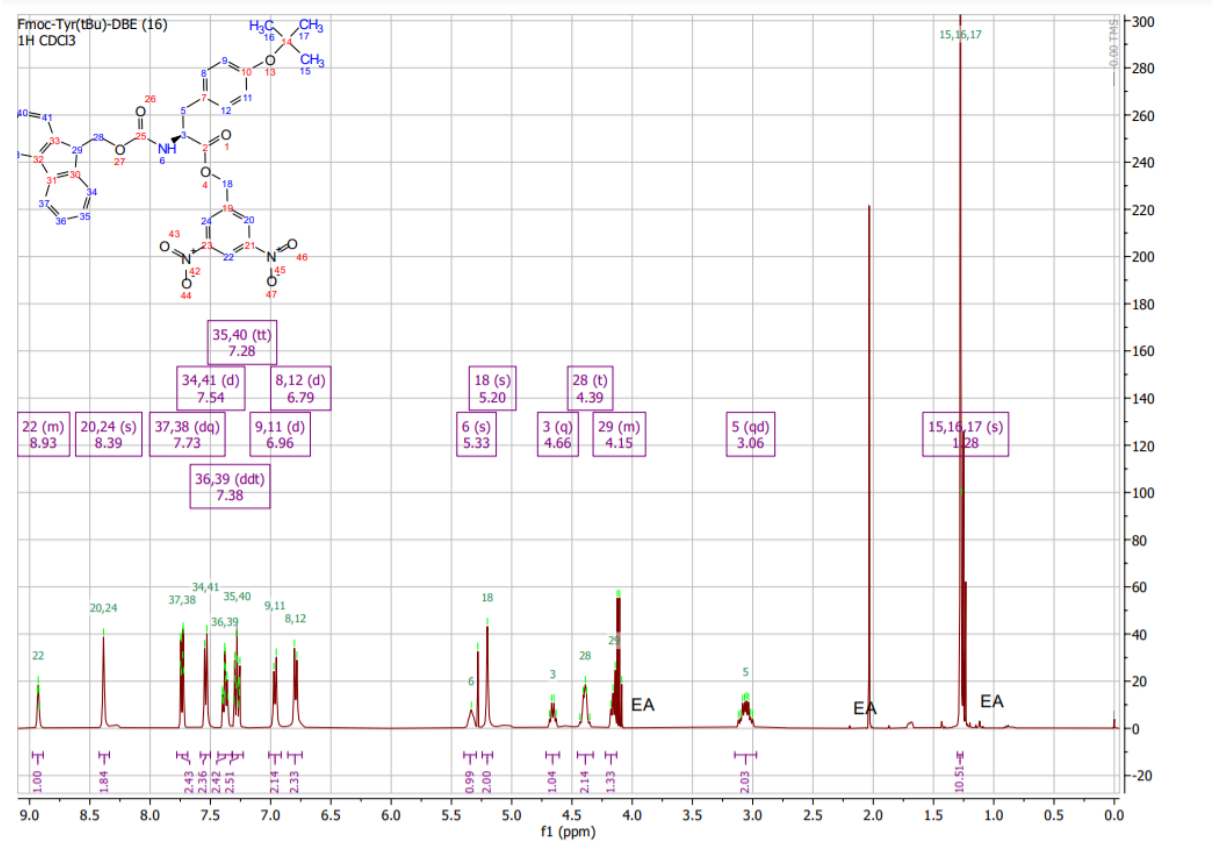
*Boc-Thr-DBE* ***(12)***

$^{1}$H NMR



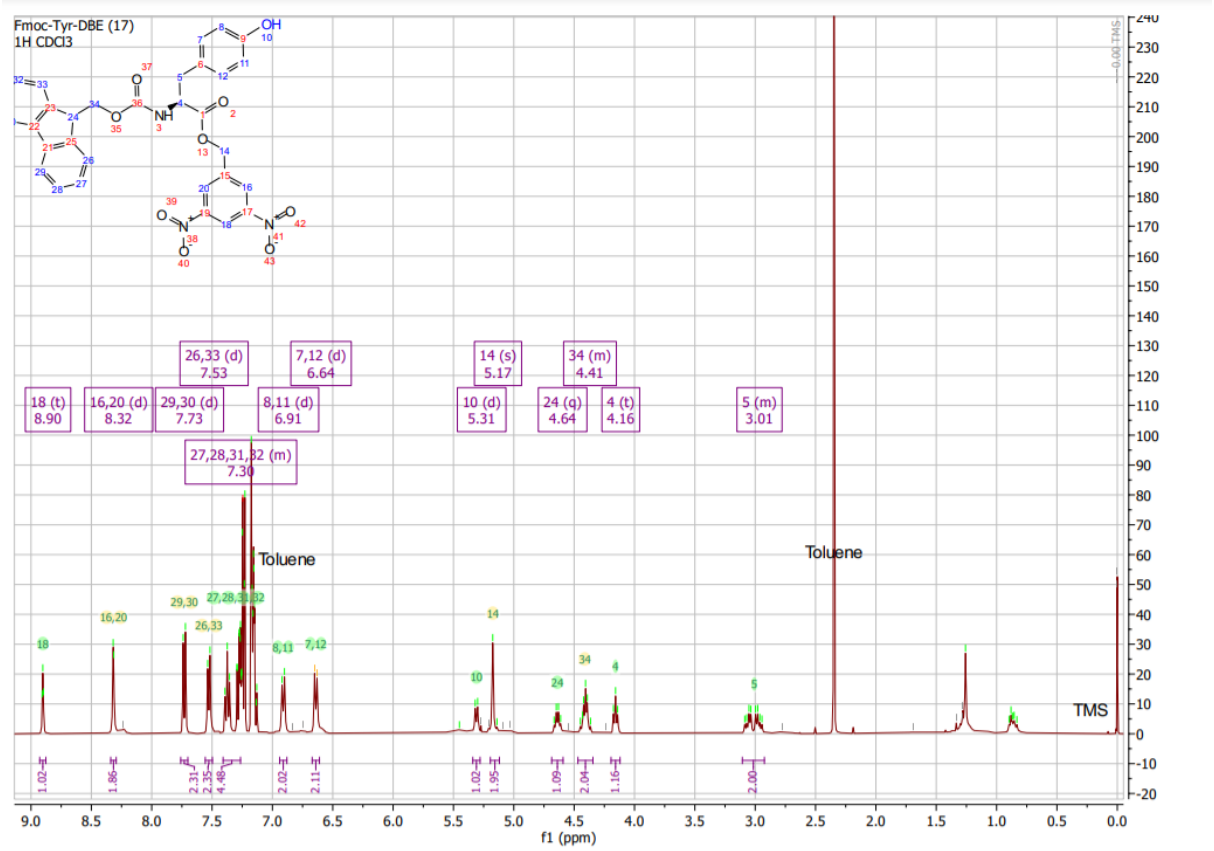
*Fmoc-Tyr(tBu)-DBE* ***(16)***

$^{1}$H NMR



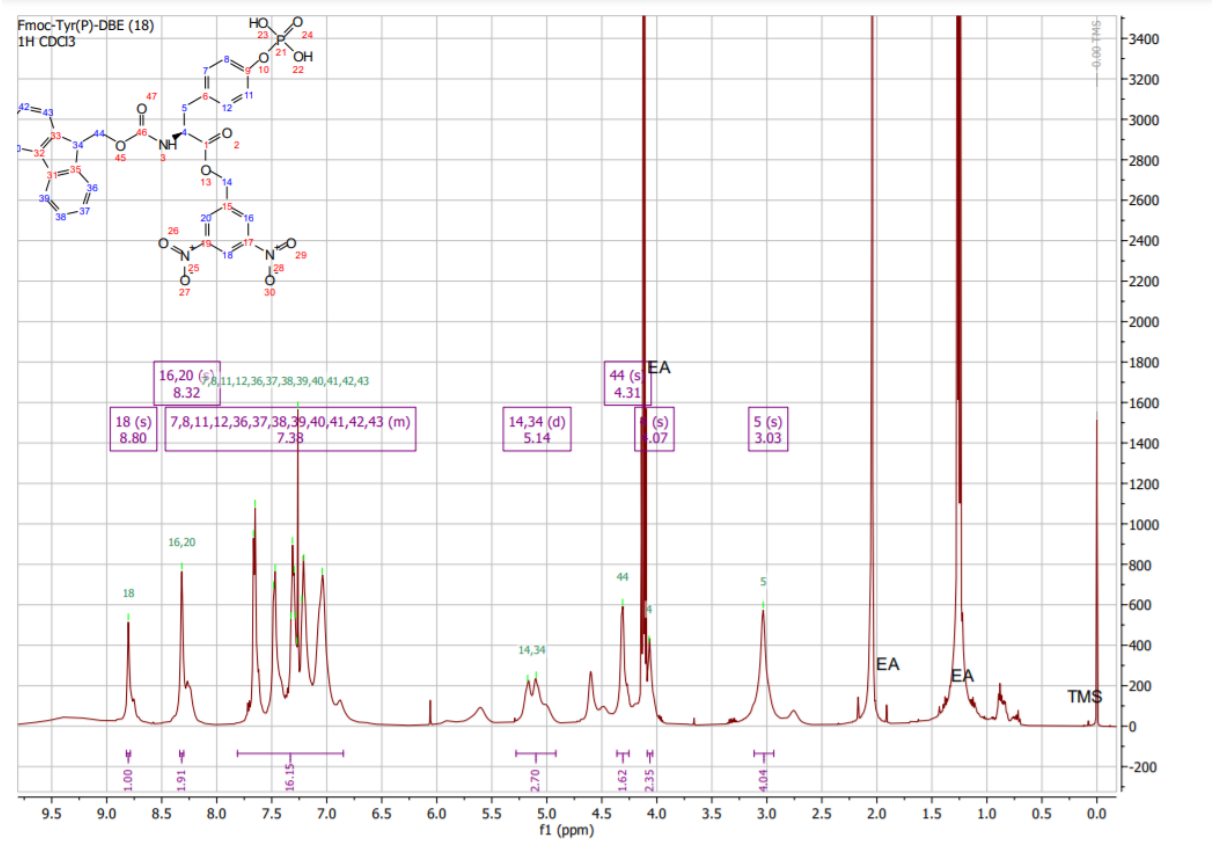
*Fmoc-Tyr-DBE* ***(17)***

$^{1}$H NMR

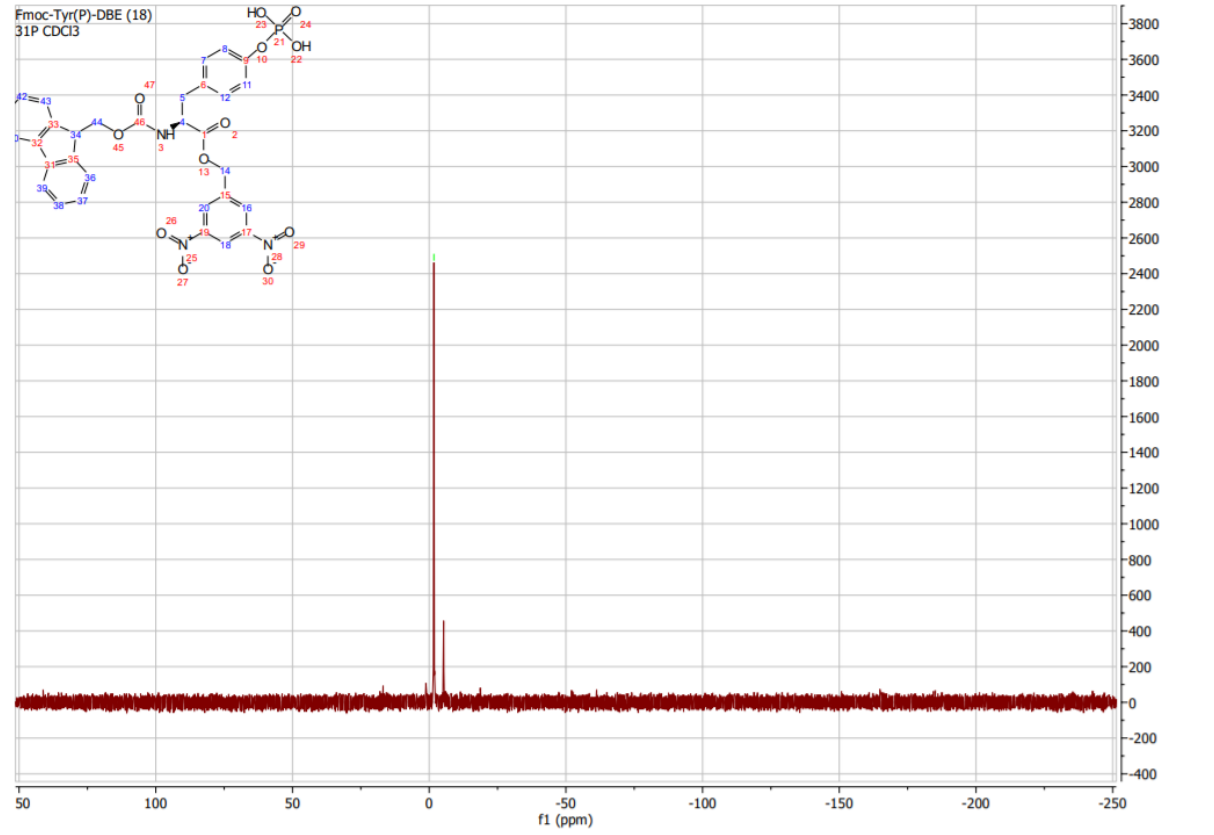


*Fmoc-Tyr(P)-DBE* ***(18)***

$^{1}$H NMR

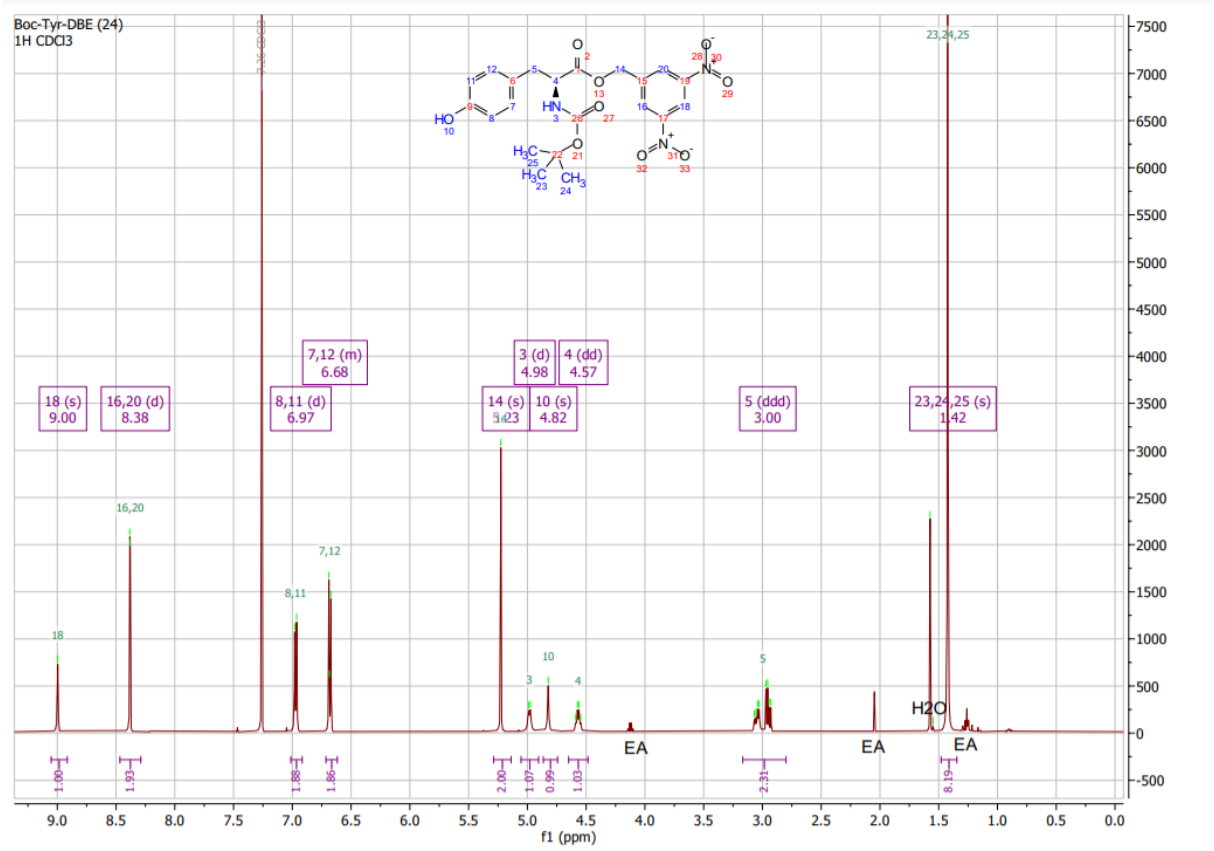



$^{31}$P NMR



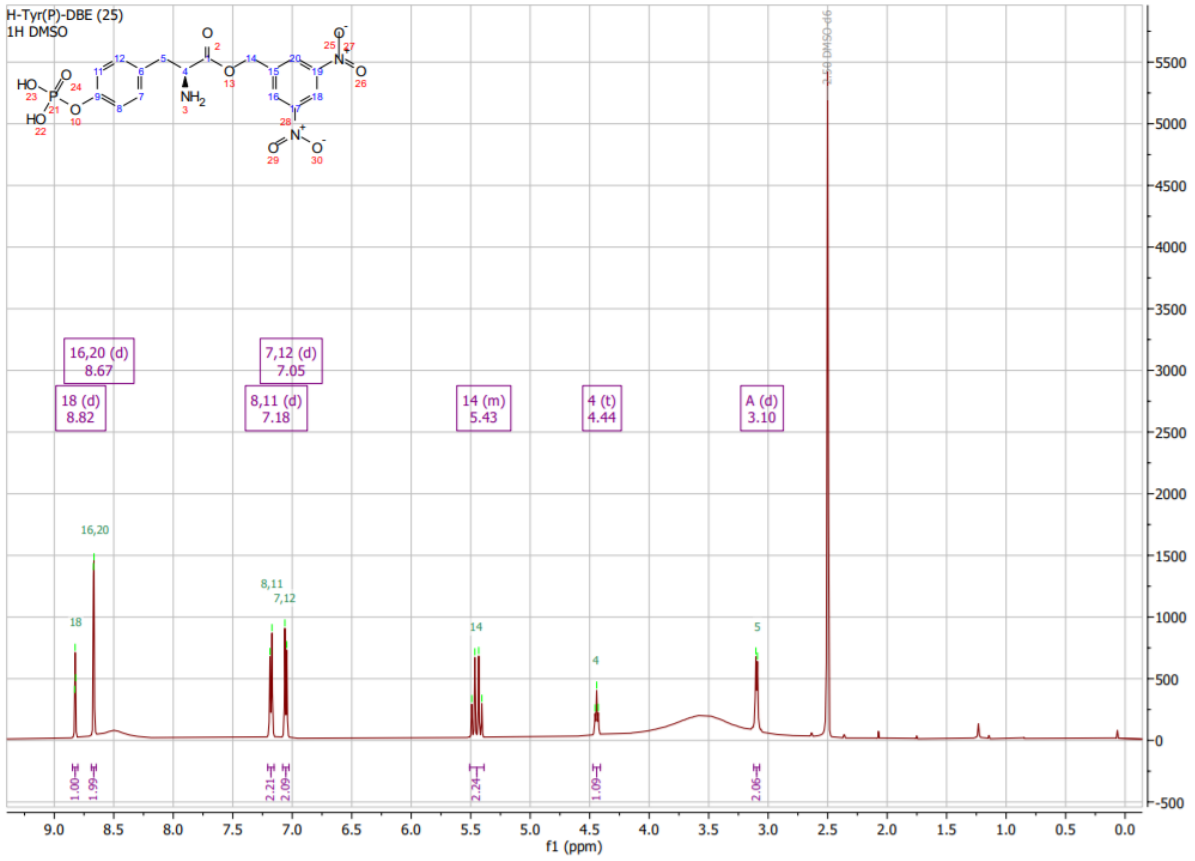
*Boc-Tyr-DBE* ***(24)***

$^{1}$H NMR

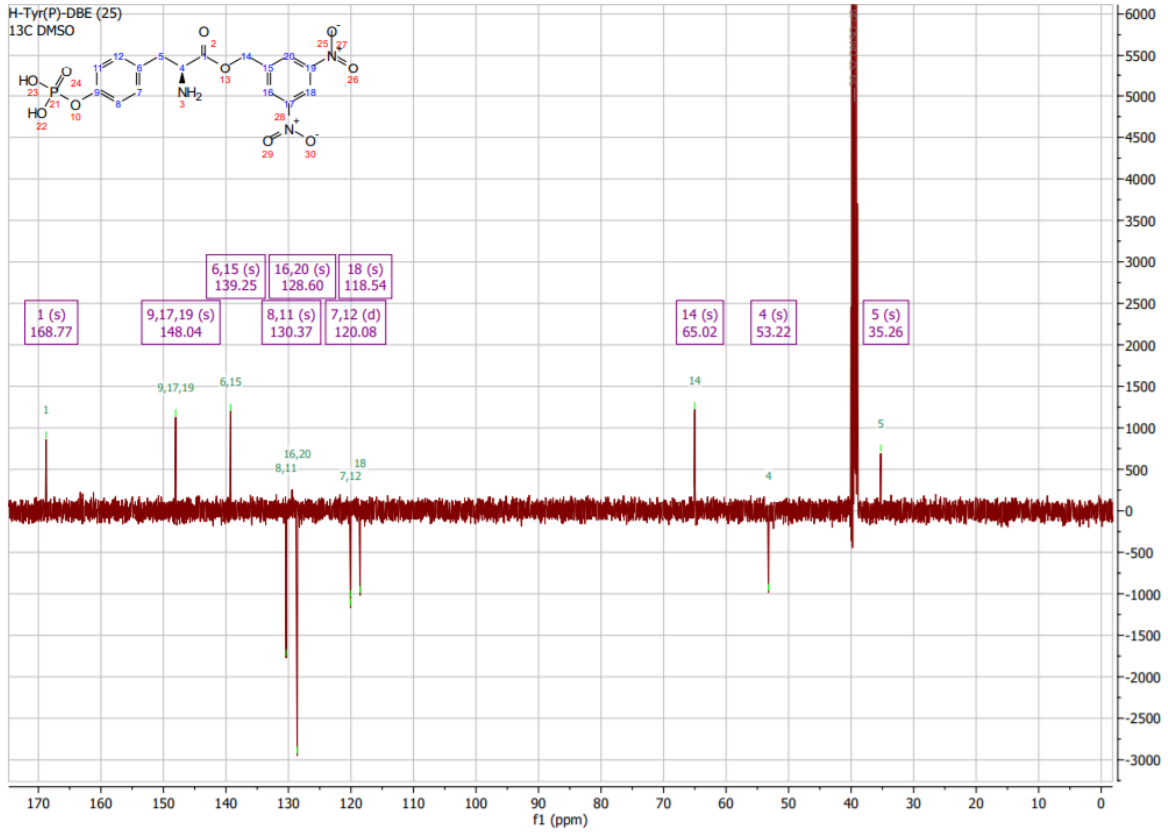


*H-Tyr(P)-DBE* ***(25)***

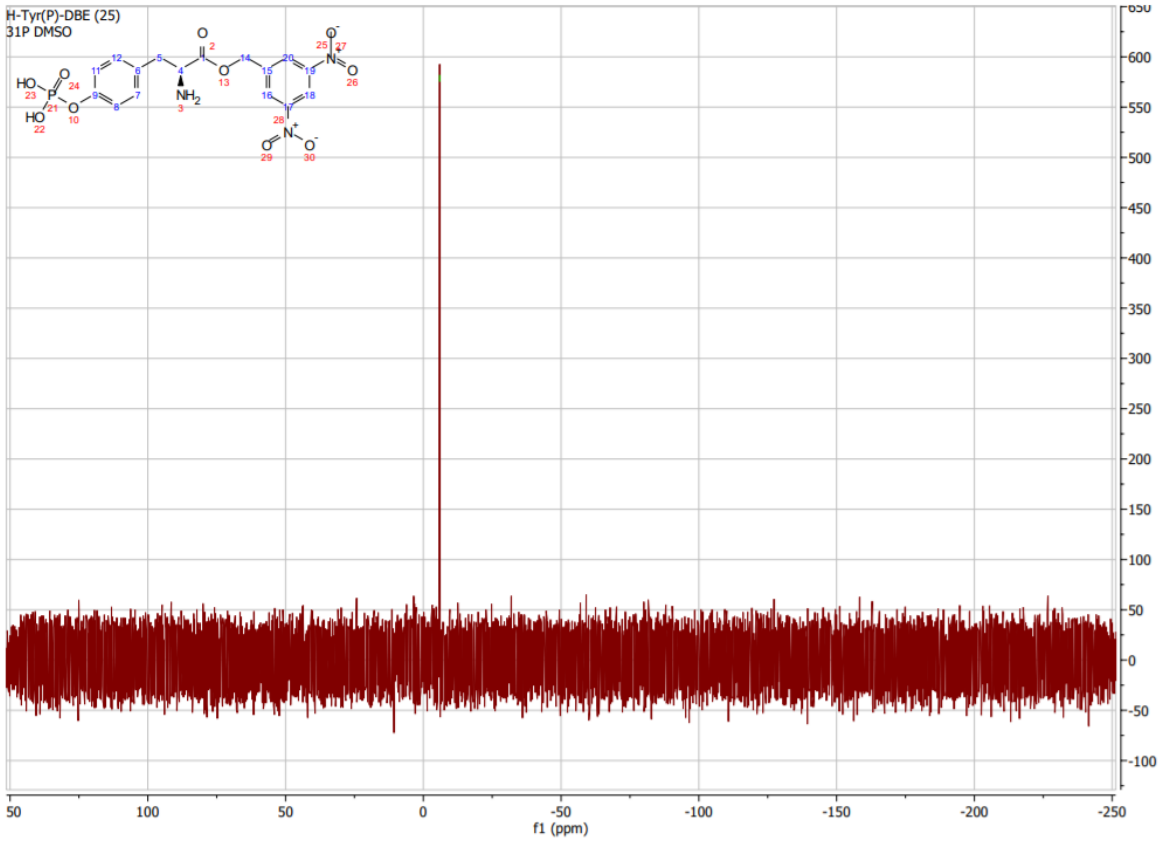
$^{1}$H NMR




$^{13}$C NMR



$^{31}$P NMR




Preparative HPLC traces