A Single-step Reaction for Glycosylation of Aminooxy Peptides

By

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Abstract

Proteins and peptides have been increasingly used to treat many diseases including autoimmune diseases and cancer. The benefits of using peptides and proteins as drugs for treatment are higher target specificity and pharmacological potency when compared to traditional small molecule drugs. However, the instability and immunogenicity of peptides and proteins can limit their translation as therapeutics. Several strategies have been used to improve the physical and chemical stability of peptides and proteins or to extend the half-life *in vivo* such as modification of *N*- and *C*-terminus, PEGylation and cyclization. In addition, glycosylation is a post-traditional modification that can improve the stability of peptides and proteins. Current approaches for synthetic glycosylation often use multiple steps, such as chemical modification of the glycans to form reactive compounds, protection of reactive groups that give undesirable products, using glycosyltransferases to transfer saccharide moieties to peptides and installation of linker molecules to improve reaction efficiency. Two different peptides, aminooxy-proteolipid peptide (AoPLP), and proteolipid peptide (PLP, a known antigen in multiple sclerosis) have been used in this report. A single-step glycosylation in aqueous buffer was achieved by reacting an aminooxy peptide to *N*-acetyl glucosamine. A series of control experiments suggested that the product formed due to the reaction of the terminal aminooxy group on the peptide with the aldehyde group that is produced from the ring opening of NAG.

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Table of contents

1. Introduction

Proteins and peptides have been increasingly used to treat many diseases including autoimmune diseases and cancer. Rituxan and Herceptin are two examples of antibodies that have been approved for the treatment of cancer. Parenteral administration is the most conventional route of delivery for protein drugs because of poor bioavailability by most other routes and greater control during clinical administration. Using peptides and proteins as drugs for treatment has advantages, such as higher target specificity and pharmacological potency when compared to traditional small molecule drugs (1). The instability and immunogenicity of peptides and proteins, however, can restrict their translation as therapeutics. Several methods have been used to improve the physical and chemical stability of peptides and proteins or to extend the half-life in vivo (1, 2, 3). Glycosylation is a post-traditional modification that can improve the stability of peptides and proteins; however, glycosylation is typically heterogeneous and results from a complex reaction pathway. New methods to synthetically glycosylate peptides and proteins are needed.

1.1 Methods to improve peptide and protein stabilities

Due to the structural complexity of the peptide and protein when compared to traditional small molecule drugs, the structural instability issues of this class of molecules generally remains one of the biggest challenges to their pharmaceutical uses (1). Therefore, different strategies have been used to

improve peptide and protein stability. These strategies include external stabilization by affecting the properties of the surrounding solvent by adding stabilizing excipients such as amino acids, sugars, polyols, metals, serum albumin and phospholipids (1, 4, 5). Also, internal stabilization has been used to improve peptide and protein stability by altering the structural characteristics of the protein through chemical modifications such as modification of N- and C-terminus, PEGylation, replacement of amino acids that are susceptible to degradation, cyclization and glycosylation (1, 4, 5).

Several proteolytic enzymes, exopeptidases, occurring in plasma, liver and kidney can remove of an amino acid from the end of a peptide chain. These enzymes are classified into two categories: (1) aminopeptidase where the cleavage of a single amino acid occurs from the amino terminal and (2) carboxypeptidase where the cleavage of a single amino acid occurs from the carboxylic end. A modification of either or both of the peptide and protein drug termini can in many cases increase enzymatic stability (2). One common example for terminal modification is N-acetylation and C-amidation. For example, it was found that C-terminal amidation and N-terminal acetylation of the immunogenic peptide MART-I27–35 prolonged the have life of the peptide by inhibiting proteolylic degradation (6).

A replacement of amino acids that are susceptible to degradation is another strategy to delay degradation and improve peptide and protein stability. For example, it was found that the half-life of luteinizing hormone releasing

hormone (LHRH) was increase from 15 min to 3.5 hours when glycine at residue six was substituted with D-leucine (7).

PEGylation is also another to method improve peptide and protein stability. Polyethylene glycols (PEGs) are mostly attached to Lys amino groups and Cys thiol groups in proteins. PEGylation of proteins can exhibit increased stability, improved solubility, decreased immunogenicity, increased circulation half-lives, and lower toxicity (8). It is known that substances with a molecular mass below 5 kDa that are not bound to plasma proteins are excreted via the renal route (1). Increasing the molecular mass of proteins can prolong the plasma half-life by inhibiting renal excretion. PEGylation can also inhibit the proteolytic degradation by masking the residues that are susceptible to degradation (9). For example, conjugation of PEG20000 to insulin increases the protein's enzymatic stability in blood (10). PEGs are generally considered to be safe and to have very low toxicity.

Moreover, cyclization of a peptide or protein is a method to inhibit proteolytic degradation and to prolong circulation half life time. For example, it was found that the half life of Growth regulating factor (GRF) was increase form 13 minutes to 2 hours when side-chain to side-chain cyclization between Asp8 and Lys12 amino acid residues was occurred (11).

1.2 Importance of glycosylation

Glycoproteins and glycopeptides are widely used in biological research and in drug and vaccine discovery. Glycosylation of proteins plays a role in protein folding, and can increase the stability of some proteins by preventing chemical reactions and physical instabilities (1). Glycosylation of peptides can produce different effects on antibody binding to the peptides. Some oligosaccharide units can be antigenic themselves and produce various anti-carbohydrate antibodies (12). However, there are also cases where antibodies recognize specific oligosaccharide structures and adjacent amino acid residues (13). In contrast to anti-carbohydrate antibodies, antibodies that recognize glycopeptidic epitopes directly do not react with free oligosaccharides. Another type of epitope occurs when antibodies recognize the epitope by its linear sequence of amino acids in a particular conformation. Glycosylation of protein is necessary for protecting the conformational structure that is required for antibody binding (13). The glycan moiety of a glycoprotein plays an important role in its pharmacokinetic properties. The structures can be involved in tissue targeting and can modulate biological activities (1). Some studies suggest that glycosylation of peptides can increase the specificity of the peptide epitope that binds to the antibody (14). Therefore, glycosylated peptides can be used in the design of vaccines. For example, it was found that increasing glycosylation of a synthetic 25-amino acid fragment of the MUCI core protein with *N*-acetylgalactosamine (GalNAc) showed greater reactivity with C595 antibody (15).

Several amino acids are susceptible to chemical degradation, such as deamidation (glutamine and asparagine), oxidation (histidine, methionine, cysteine, tryptophan, and tyrosine), and disulfide fragmentation (cysteine). Peptide hydrolysis can be produced either by proteases or by pH sensitive backbone sequences, at Asp-Gly and Asp-Pro (1, 8). Several studies show that glycosylation can also improve proteolytic stability of peptides and proteins. This proteolytic stability results from steric hindrance that is provided by glycans such as NAG, galactose, and sialic acid (1, 16). Moreover, glycosylation can improve the stability of peptides and proteins by inhibiting oxidation. Oxidation of peptides and proteins can occur in several amino acid side chains such histidine, methionine, cysteine, tryptophan, and tyrosine. When a protein formulation is exposed to ultraviolet light in the presence of oxygen, the protein can be oxidized and form undesirable products. For example, the deglycosylated form of erythropoietin was found to be more susceptible to oxidation than the naturally glycosylated erythropoietin (17). Chemical instability due to either disulfide or non-disulfide crosslinking can also be prevented by glycosylation. Formation of these covalently linked disulfide species in proteins can lead to loss of bioactivity and formation of insoluble protein aggregates. Several studies suggest that the presence of glycan at the protein surface provides intermolecular steric repulsion between the protein species prone to crosslinking (1).

Glycosylation can also provide physical stability. It is known that hydrophobic residues are located inside the protein core to avoid exposing them

to aqueous solution, giving rise to a compact native state. Several types of interactions, such as electrostatic, charge-charge interactions, hydrogen bonds, and Van der Waals interactions stabilize the native state. The conformational stability of folded proteins is very sensitive to the environmental conditions such as temperature, pH, ionic strength, and the presence of chemical denaturants. Changing the temperature, pH, or ionic strength can lead to changes in protein conformation and can produce unfolded structures. It was found that glycosylation could increase the stability of proteins against changes in pH or temperature, and against the effects of chemical denaturants, such as urea and guadinidium hydrochloride (1). It was also found that glycosylation can improve the solubility of proteins by providing favorable interactions between the surface of the glycoprotein and the solvent molecules (1).

1.3 Types of glycosylation

Glycosylation takes place in the endoplasmic reticulum (ER) and golgi apparatus and is catalyzed by the combination of glycosyltransferases and glycosidases present in the cell (18, 19, 20). It was estimated that at least 50% of all mammalian proteins are glycosylated (21). Many protein residues have been found to be glycosylated by different glycans such as fucose, galactose, mannose (Man), NAG, *N*-acetylgalactosamine, and sialic acid (*N*-acetylneuraminic acid) (22, 23).

Glycosylation is classified by type based on the atom of the amino acid that binds to the glycan. In *N*-glycosylation, glycans attach to the side chain nitrogen of asparagine in the sequence containing Asn-Xxx-Ser/Thr, where Xxx corresponds to any amino acid except for proline (1, 24). In *O*-glycosylation, glycans attach to the side chain hydroxyl oxygen of serine, threonine or tyrosine. This type of glycosylation does not require a unique sequence such as in the case of *N*-linked glycans (1). There is also another rare type of glycosylation, which is called *C*-glycosylation, where a glycan can attach to a carbon on a tryptophan side-chain. The structure of protein-linked oligosaccharide chains depends on several factors involved in the biosynthetic machinery of the cell, such as the precence of glycosyltransferases and other enzymes, the availability of oligosaccharides and the presence of the protein or glycoprotein acceptor in the cell (24).

1.4 Reaction of aminooxy compounds with NAG

It is known that aminooxy groups are more reactive than primary amines. The reactivity of the aminooxy group could be due to the lower pKa of the aminooxy group (pKa = 5-6) as compared to an amino group (pKa ~9). This keeps the aminooxy group unprotonated at lower pH and makes it a better nucleophile. Our group found that there appeared to be a reaction between the aminooxy peptides and the *N*-acetyl amide group of NAG. Mass spectroscopy data indicated that there was a reaction product between NAG and *O*-

(carboxymethyl) hydroxylamine (OCMH) (Figure 1). The H1 NMR data (Figure 2) and C13 NMR (Figure 3) confirmed that the reaction occurred. In the H1 NMR, product spectra showed the appearance of peaks at ~6.5 and 7.5 ppm corresponding to the change in the environment caused by the product. In C13 NMR, the product spectra showed that the amide carbon peak (~175 ppm) shifted to ~150 ppm corresponding to the product formation. In addition, the aminooxy reactive peptide can be conjugated to hyaluronic acid (HA), which is a polymer chain containing NAG. Although the data suggested that a reaction occurred, the product formed and reaction kinetics have not been thoroughly studied and the reaction has not been characterized and optimized.

Due to the importance of glycosylation on the physical and chemical stability of peptides and proteins, simple synthetic glycosylation approaches are needed. Aminooxy chemistry may provide a viable option as evidenced by our preliminary studies. Here, the reaction kinetics of adding NAG to two different peptides, aminooxy-proteolipid peptide (AoPLP), and proteolipid peptide (PLP, a known antigen in multiple sclerosis) with an amine terminus was studied, and the reactivity of these peptides was compared. The reaction conditions were examined at various pH and buffer.

2. Materials and Methods

2.2 Materials

N-acetyl glucosamine, 3-O-Methyl-N-acetyl-D-glucosamine, benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside, acetanilide, O-allyl-hydroxylamine, *O*-Benzyl-hydroxylamine, O-(carboxymethyl) hydroxylamine hemihydrochloride (OCMH), O-methylhydroxylamine and Anthranilic acid were purchased from Sigma. Amino acids were purchased from Peptides International. Analytical grade acetonitrile, sodium cyanoborohydride, research grade sodium acetate, acetic acid, citric acid, sodium citrate monosodium phosphate, disodium phosphate colchicine and synthesis grade trifluoro acetic acid (TFA) were purchased from Fisher Scientific. Peptides were synthesized in house. Water was offered by a Labconco Water PRO PS ultrapure water purification unit.

2.2 Methods

2.2.1 Peptide synthesis

Peptides were synthesized using 9-fluorenylmethyloxy-carbonyl-protected amino acid chemistry on polyethylene glycol-polystyrene resins. The peptides synthesized were *aminooxy-proteolipid peptide* (AoPLP) and *proteolipid peptide* (PLP) (Figure 4), which is known to be an antigen epitope in multiple sclerosis. Peptides were cleaved from the resin, and the crude peptides were isolated by precipitation in ether. Peptides were purified by high performance liquid chromatography (HPLC) using a C18 column. Analytical HPLC and mass

spectroscopy (MS) were used to calculate purity and confirm the identity of the synthesized peptides.

2.2.2 Reaction of peptides (AoPLP and PLP) with NAG and other compounds

Three milligrams of each peptide (1.9 mM of AoPLP and 2 mM of PLP) were mixed with 1.7 mg of NAG (8 mM) in 1 mL of acetate buffer (50 mM), pH 5. After addition of all reactive species the pH was measured and adjusted to 5. The reactions were run for 17 hours at room temperature. For AoPLP and PLP, three different pH values have been used: pH 5 acetate buffer (50 mM), pH 6 citrate buffer (50 mM), and pH 7 phosphate buffer (50 mM). Moreover, four different amide compounds, benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside, colchicine, acetanilide and 3-O-methyl-N-acetyl-D-glucosamine (Figure 5) have been used to examine the potential reactivity of aminooxy peptide (AoPLP) with amide groups. For colchicine and 3-O-Methyl-N-acetyl-D-glucosamine, the reaction conditions were similar to those of the previous reaction. However, benzyl-2-acetamido-2-deoxy-α-D-galacto-pyranoside and acetanilide are only partially soluble in water. Therefore, acetonitrile-acetate buffer (40:60) was used to increase the solubility. After addition of all reactive species the pH was measured and adjusted to 5. The reactions were run for 17 hours at room temperature.

2.2.3 Investigation the reacting site

This experiment has been carried out to examine the site of reaction of aminooxy groups. Different aminooxy compounds, (O-allyl-hydroxylamine, *O*-benzyl-hydroxylamine, O-(carboxymethyl) hydroxylamine hemihydrochloride (OCMH) and O-methylhydroxylamine) solutions have been prepared with concentration of 0.25 M in distilled water. 34.25 mg of Anthranilic acid (0.25 M) and 31.42 of sodium cyanoborohydride (0.5 M) were dissolved in 1ml of 4% (w/v) sodium acetate trihydrate and 2% (w/v) of boric acid in methanol (mixture I). Also, 31.42 mg of sodium cyanoborohydride was dissolved in 1ml of 4% (w/v) sodium acetate trihydrate and 2% (w/v) of boric acid in methanol (mixture II). 55.3 mg of NAG (0.25 M) was dissolved in 1 ml of distilled water. 40 μL of NAG solution was mixed with 40 μL of mixture I in four different tubes and 2 μL of acetic acid was added in each tube. The reaction was carried out at 80 ^{0}C for one hour. After that the reaction mixture was cooled at room temperature for one hour. 40 μL of each aminooxy solution was added to each tube. The reactions were carried out at room temperature for 17 hour followed by lyophilization. Electrospray ionization mass spectrometry was used to confirm the presence of any products. In other four different tubes, 40 μL of NAG solution was mixed with 40 μL of mixture II. 40 μL of each aminooxy solution was added to each tube and 2 μL of acetic acid was added in each tube. The reaction was carried out at 80 ^{0}C for one hour. Electrospray ionization mass spectrometry was used to confirm the presence of the product.

2.2.4 Mass Spectroscopy

Electrospray ionization mass spectroscopy was used to determine the masses of synthesized peptides and the masses of conjugated peptides using a waters LCT premier ESI mass spectrometer running MassLynx software.

Matrix assisted laser desorption/ionization (MALDI)-TOF was also used to determine the masses of conjugated peptides. MALDI-TOF MS spectra were acquired using a Voyager DE-STR mass spectrometer equipped with delayed extraction. An acceleration voltage of 20 kV and a nitrogen laser at 337 nm was used. Mass spectra were acquired in positive reflector mode. External calibration was used. Cyano-4-hydroxycinnamic acid was used as matrix.

2.2.5 High Performance Liquid Chromatography

Peptide was quantified by gradient reversed phase HPLC (SHIMADZU) using a Vydac HPLC protein and peptide C18 column. The HPLC is composed of a SCL-20A SHIMADZU system controller, LC-10AT VP SHIMADZU liquid chromatograph, SIL-10A XL SHIMADZU auto-injector set at 75 μL injection volume, DGU-14A SHIMADZU degasser, sample cooler, and SPD-10A SHIMADZU UV-vis detector (220 nm). The HPLC-UV system was controlled by a personal computer equipped with SHIMADZU class VP Software. Gradient elution was carried out at constant flow of 1 mL/min, from 100% A to 35% A (corresponding to 0% B to 65% B) for 50 min, followed by an isocratic elution at

75% B for 3 min. Mobile phase (A) was acetonitrile-water (5:95) with 0.1% TFA and mobile phase (B) was acetonitrile with 0.1% TFA.

2.2.6 Size Exclusion Chromatography

Size exclusion chromatography was used to isolate the product using Superdex Peptide 10/300 GL column, eluted isocratically. Three different mobile phases, (1) 0.1 M ammonium acetate at flow rate of 0.5 ml/min, (2) acetonitrile-water (10:90) with 0.1% T trifluoroacetic acid (TFA) at flow rate of 0.5 ml/min, and (3) 0.1 M sodium phosphate at flow rate of 0.4 ml/min have been tried. The HPLC consisted of a SCL-20A SHIMADZU system controller, LC-10AT VP SHIMADZU liquid chromatograph, SIL-10A XL SHIMADZU auto-injector set at 200 μL injection volume, DGU-14A SHIMADZU degasser, sample cooler, and SPD-10A SHIMADZU UV-vis detector (220 nm). The HPLC-UV system was controlled by a personal computer equipped with SHIMADZU class VP Software.

3. Results and discussion

3.1 Peptide synthesis

After the synthesized peptides were purified by high performance liquid chromatography (HPLC) using a C18 column, the identity of the synthesized peptides was confirmed by electrospray ionization mass spectroscopy. The molecular weights for AoPLP and PLP were 1593.81 and 1520.7, respectively (Figure 6 and 7). The purity of the synthesized peptides was calculated by

gradient reversed phase HPLC using a Vydac HPLC protein and peptide C18 column. The purities of AoPLP and PLP were 89% and 97% respectively (Figure 8 and 9).

3.2 Reaction of the peptides (AoPLP and PLP) with NAG

The reaction of PLP (Mw 1521.76) with NAG (Mw 221) was carried out at pH 5 in acetate buffer (50mM) for 17 hours. The expected molecular weight of the product of the reaction of amino terminus group with reducing end of NAG is 1724. The product was analyzed by mass spectroscopy. The spectrum for the reaction product between PLP and NAG showed the absence of the product (Mw 1724) at pH 5 (Figure 10)

The reaction of AoPLP (Mw 1594.8) with NAG (Mw 221) has been carried out under similar conditions. The spectrum for the reaction product between AoPLP and NAG (Figure 11) showed the presence of the product (Mw 1798). The product was also detected after reacting at pH 6 (Figure 12) and 7 (Figure 13). The reactivity of the aminooxy group can be due to the lower pKa of the aminooxy group (pKa = 5-6) as compared to amino group (pKa ~9). This keeps the aminooxy group unprotonated at lower pH and makes it a better nucleophile. The formed product could be due to the reaction of the aminooxy group on the peptide with the amide group on NAG or with the aldehyde group that is produced from the ring opening of NAG. Therefore, different amide compounds have been used to examine the reactivity of aminooxy group with amide group.

The reaction between AoPLP and NAG was also carried out at pH 6 in citrate buffer (50 mM) and pH 7 in phosphate buffer (50 mM).

The reaction has been carried out in aqueous buffer, thus avoiding harsh catalysts or reaction conditions. In addition, the aminooxy group is significantly more reactive than primary amines, thus conferring the desired specificity for glycosylation of peptides (25, 26). Simplified reaction schemes would be a benefit for synthetic glycosylation. Due to stability concerns, peptides and proteins cannot be exposed to many of the conditions that are required for glycosylation such as organic solvent, high or low pH, and temperature (27). In current approaches, multiple steps are often used for synthetic glycosylation, such as chemical modification of the glycans to form reactive compounds, protection of reactive groups that give undesirable products, using glycosyltransferases to transfer saccharide moieties to peptides and installation of linker molecules to improve reaction efficiency (28, 29,30). Some of these steps need to be carried out at harsh conditions that may be harmful for the synthesis of glycoproteins, and consequently, often afford fairly poor yields.

For example, Thomas J. Styslinger's group has developed a strategy for glycosylation of hemoglobin with variable molecular weight oligosaccharides. This strategy involves three steps for glycosylation of hemoglobin. In the first step, an aminooxy group on polyethylene glycol (PEG) was reacted with the aldehyde available in the open-chain conformation of the reducing end carbohydrate. The second step involved installation of a reactive terminal

functional group on PEG. In the third step, thiol-targeting chemistry was used to successfully conjugate glycans to a cysteine residue on the hemoglobin (29). These inconveniences and lengthy processes make many of the current glycosylation methods overly complicated for therapeutic glycoproteins and unrealistic for scale-up (28, 31). In this thesis, a single-step glycosylation in aqueous buffer was achieved by reacting an aminooxy peptide directly to *N*-acetyl glucosamine.

3.3 Size exclusion chromatography

Size exclusion chromatography was used to isolate the product. Different mobile phases were tested: 0.1 mM ammonium acetate at flow rate 0.5 ml/min and volume of injection of 200 μL, acetonitrile-water (10:90) with 0.1% trifluoroacetic acid (TFA) at flow rate 0.5 ml/min and volume of injection of 200 μL, and 0.1 mM sodium phosphate at flow rate 0.4 ml/min and volume of injection of 100 μL. When ammonium acetate (Figure 14) and acetonitrile-water (Figure 15) were used as mobile phases, the separation of the peaks was sufficient to isolate the product. The separation of the peaks was better when using sodium phosphate as mobile phase (Figure 16). Each of the three peaks was identified by injecting free peptide (AoPLP) and free NAG. The first peak was the product (retention time = 37 min), the second peak was AoPLP (retention time = 39.8 min), and the third peak was NAG (retention time = 42.7 min).

3.4 Examine the reactivity of amide group

There are two proposed pathways for forming the product. The product formed could be due to the reaction of the terminal aminooxy group on the peptide with the amide group on NAG or with the aldehyde group that is produced from the ring opening of NAG. Therefore, four different amide compounds, benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside (Mw 311.3), colchicine (MW 399.4) acetanilide (MW 135.16) and 3-O-methyl-N-acetyl-D-glucosamine (MW 235.23) were used to probe the reactivity of the aminooxy group (AoPLP) with the carbonyl of the amide group. For colchicine, and 3-O-methyl-N-acetyl-D-glucosamine, the reaction conditions were similar to those of the previous reaction. However, benzyl-2-acetamido-2-deoxy-α-D-galacto-pyranoside and acetanilide were only partially soluble in water. Therefore, acetonitrile-acetate buffer (40:60) was used to increase the solubility.

For the reaction of AoPLP with benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside (expected product mass is 1886.8) (Figure 17) with colchicine (expected product mass is 1975) (Figure 18), with acetanilide (expected product mass is 1710.9) (Figure 19) and with 3-O-methyl-N-acetyl-D-glucosamine (expected product Mw 1810.9) (Figure 20) the mass spectrum for the reaction products showed the absence of the products. The mass spectrum data indicated that there was no reaction between AoPLP and the four amide compounds. The mass spectrum data may suggest that the product that is formed from the reaction of AoPLP with NAG is due to the ring opening of NAG.

There are some studies suggested that the product that is formed is due to the ring opening of NAG. For example, conjugation of florescent probes that have an amino group such as 2-aminobenzamide and anthranilic acid with protein-drivative glycans (32). Due to the low intrinsic spectral activity and detection of glycan during chromatographic processes, this fluorescent labeling technique was used.

3.5 Investigation of the NAG aldehyde reaction site

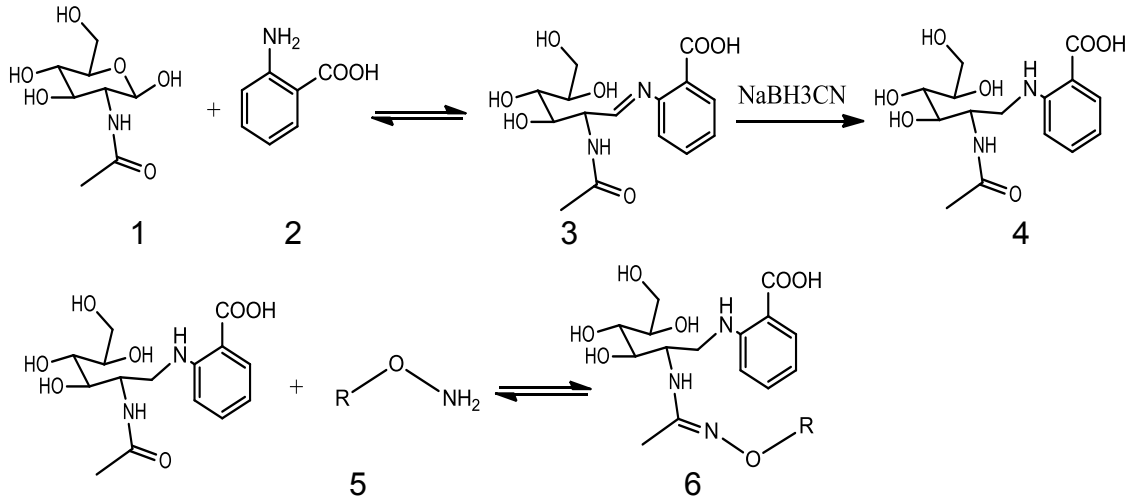
In this experiment, the aldehyde group that is formed from the ring of NAG was blocked by reacting it with the amino group of anthranilic acid. Sodium cyanoborohydride was added to reduce the imine group forming secondary amine and making the reaction irreversible. Therefore, the reaction will be pushed toward the product. The expected mass for the product that results from the reaction of NAG wih anthranilic acid after reducing the imine bond is 342.1. After that, different aminooxy compounds O-allyl-hydroxylamine, *O*-benzyl-hydroxylamine, O-(carboxymethyl) hydroxylamine hemihydrochloride (OCMH), and O-methylhydroxylamine were added to investigate the reactivity of amide group (Scheme 1). The expected masses for products that would result from the reaction of amide group of the blocked NAG with the aminooxy group of O-methylhydroxylamine, O-allyl-hydroxylamine, *O*-Benzyl-hydroxylamine, and or O-(carboxymethyl) hydroxylamine hemihydrochloride (OCMH) are 371.2, 397.2, 447.2, and 415.2, respectively. The mass spectrum data indicated that there was

a reaction between NAG and anthranilic acid. However, the mass spectrum data indicated that there were no reactions between the blocked NAG and the aminooxy compounds (Figure 21, 22, 23 and 24).

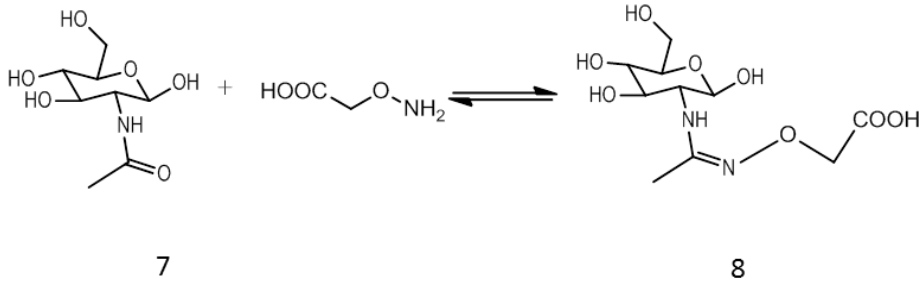
In another experiment, the reactions between NAG and aminooxy compounds were carried out in the presence of a reducing agent (sodium cyanoborohydride). The expected masses for the products that resulted from the reaction of NAG with the aminooxy group of *O*-methylhydroxylamine, *O*-allyl-hydroxylamine, *O*-benzyl-hydroxylamine, and *O-(*carboxymethyl) hydroxylamine hemihydrochloride (OCMH) without reducing the oxime group are 250.13, 276.13, 326.15 and 294.1063 and after reducing reducing the oxime bond are 252.14, 397.14, 328.16 and 294.12, respectively. The mass spectrum data indicated that there were reactions between the NAG and the aminooxy compounds (Figure 25, 26, 27 and 28). Both of experiments suggest that the product that was formed from the reaction of AoPLP with NAG was due to the ring opening of NAG. After blocking the aldehyde group that was produced from the ring opening of NAG, the reactions of aminooxy compounds with NAG were prevented. Fourier Transform Infrared Spectroscopy (FTIR) can also be used to investigate the reaction site by looking at the change on the amide bonding environments at 1650 and 1550 cm-1 throughout the course of the reaction of aminooxy compound and NAG (33).

4. Conclusion

In this study, two different peptides, AoPLP and PLP, were used to probe the reactivity with NAG. It was found that there was no reaction between PLP and NAG at pH 5. However, the reaction between AoPLP and NAG was observed at pH 5, 6 and 7. Two different experiments were used to investigate the reacting site (peptide with the amide group on NAG or with the aldehyde group that is produced from the ring opening of NAG) of the aminooxy peptide (AoPLP). In the first experiment, AoPLP was reacted with four different amide compounds. The mass spectra data indicated that there was no reaction between the aminooxy peptide and the amide compounds. In the second experiment, the aldehyde group that is produced from the ring opening of NAG was blocked by reacting it with anthranilic acid. After that, the blocked NAG was reacted with different aminooxy compounds. The mass spectra data indicated that there was no reaction between the aminooxy compounds and the blocked NAG. However, when the aminooxy was reacted with free NAG, the product that resulted from the reaction of aminoxy group with the aldehyde group was observed. These experiments also suggested that the product that was formed from the reaction of AoPLP with NAG was due to the ring opening of NAG. In this report, a single-step glycosylation was achieved by reacting an aminooxy peptide to *N*-acetyl glucosamine.



Scheme 1: Proposed reaction scheme for the NAG aldehyde reaction site. 1. NAG. 2. Anthranilic acid. 3. Labeled NAG without reducing the oxime bond. 4. Labeled NAG after reducing the oxime bond. 5. Aminooxy compounds. 6. A possible NAG reaction productat the amide carbonyl carbon.



Scheme2: Proposed reaction scheme for the reaction of NAG and OCMH.

7. OCMH. 8. A possible amide reaction product.

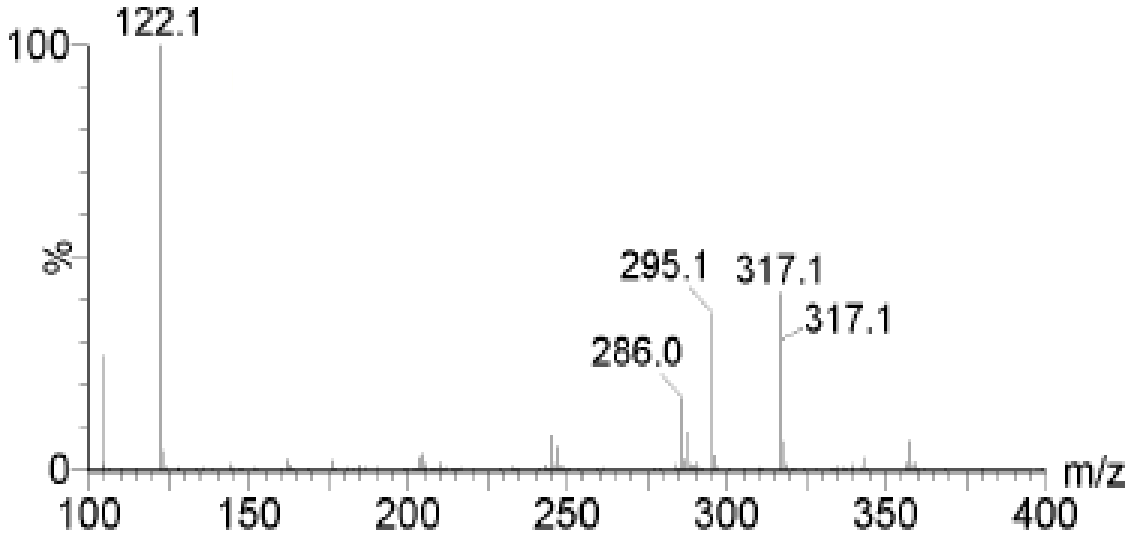


Figure 1: ESI+ Mass spectroscopy of the unpurified reaction product between NAG and OCMH showing the product peak at 295 and product plus Na+ at 317 (Joshua Sestak's thesis).

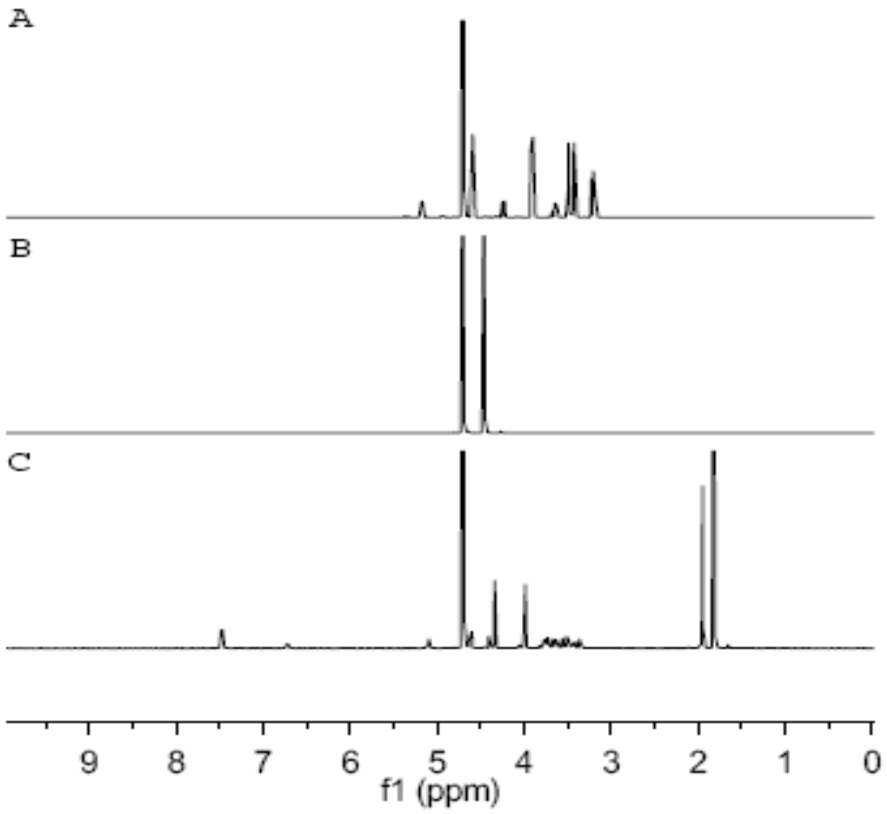


Figure 2: H1 NMR spectrum of (A) NAG, and (B) OCMH, (C) reaction product of NAG monomer with OCMH (Joshua Sestak's thesis).

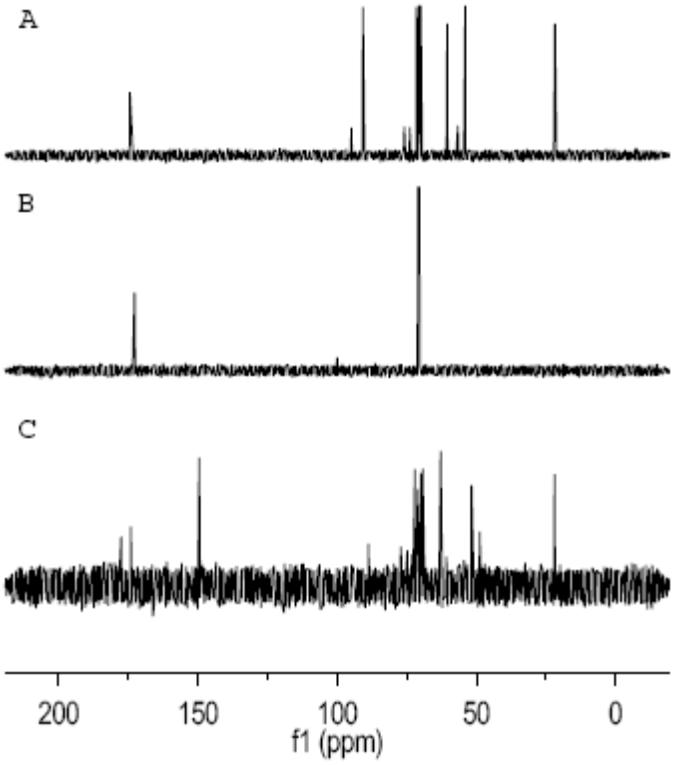
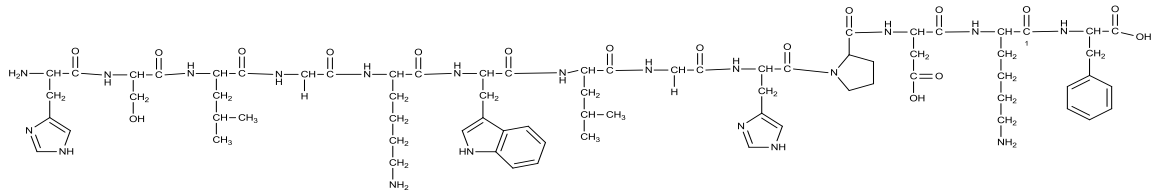
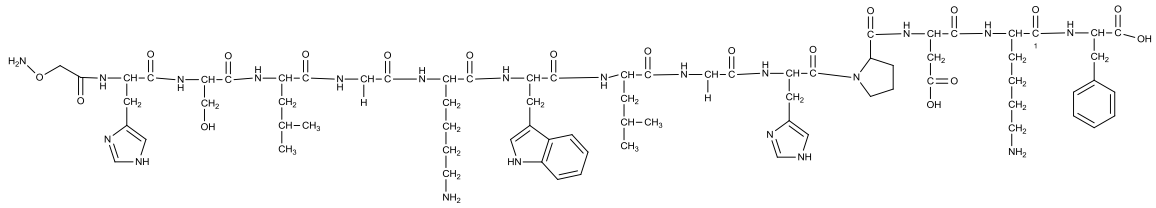


Figure 3: C13 NMR spectrum of (A) NAG monomer, (B) OCMH, (C) reaction product of NAG monomer and OCMH (Joshua Sestak's thesis).

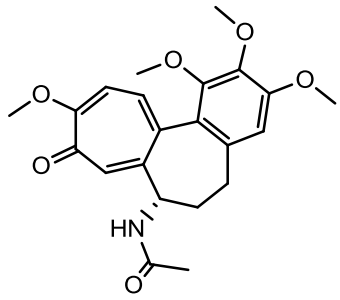


(a)

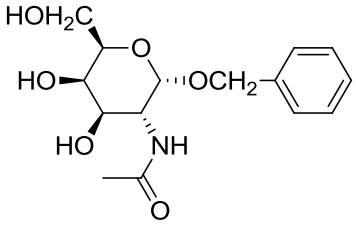


(b)

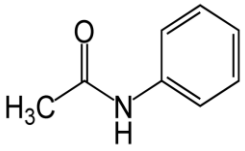
Figure 4: The structures of (a) PLP and (b) AoPLP.



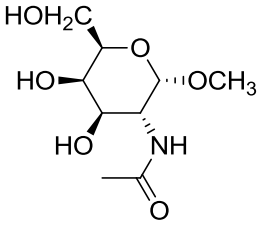
(a)



(b)



(c)



(d)

Figure 5: The structures of (a) colchicine, (b) Benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside, (c) acetanilide, (d) 3-O-Methyl-N-acetyl-D-glucosamine**.**

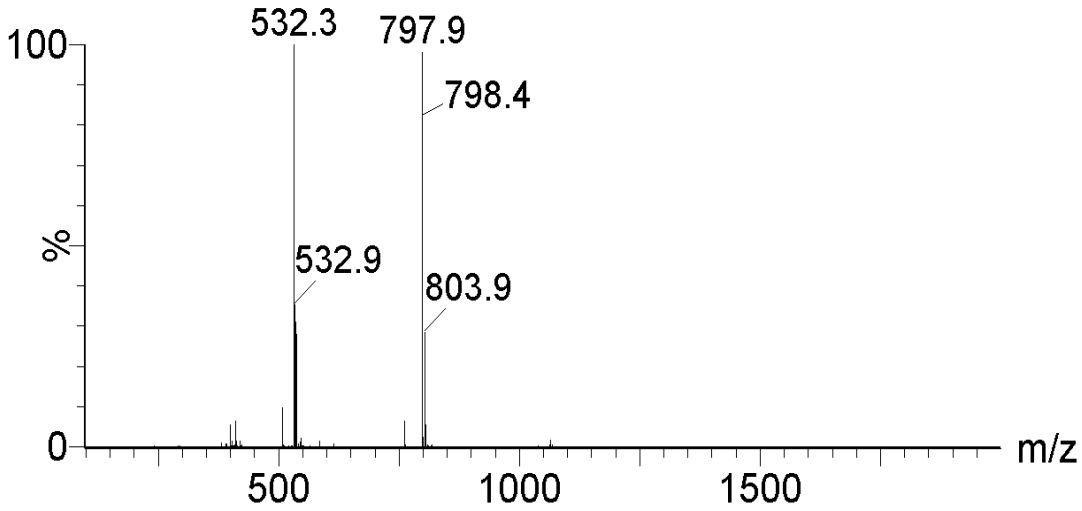


Figure 6: ESI+ mass spectroscopy of synthesized AoPLP peptide showing the peaks at 532.3 (AoPLP peptide (+3)) and at 797.9 (AoPLP peptide (+2)).

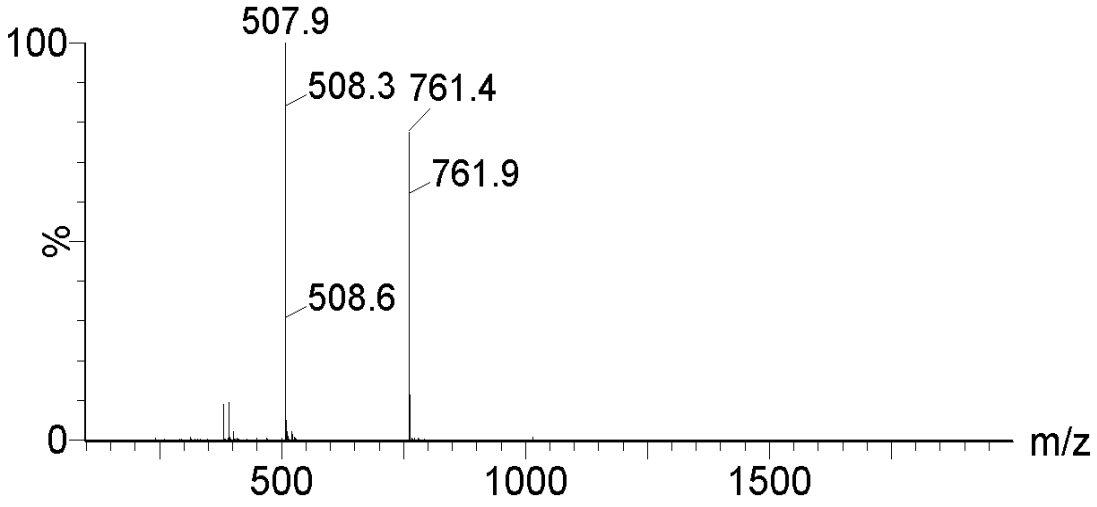


Figure 7: ESI+ mass spectroscopy of synthesized PLP peptide showing the peaks at 507.9 (PLP peptide (+3)) and at 761.4 (PLP peptide (+2)).

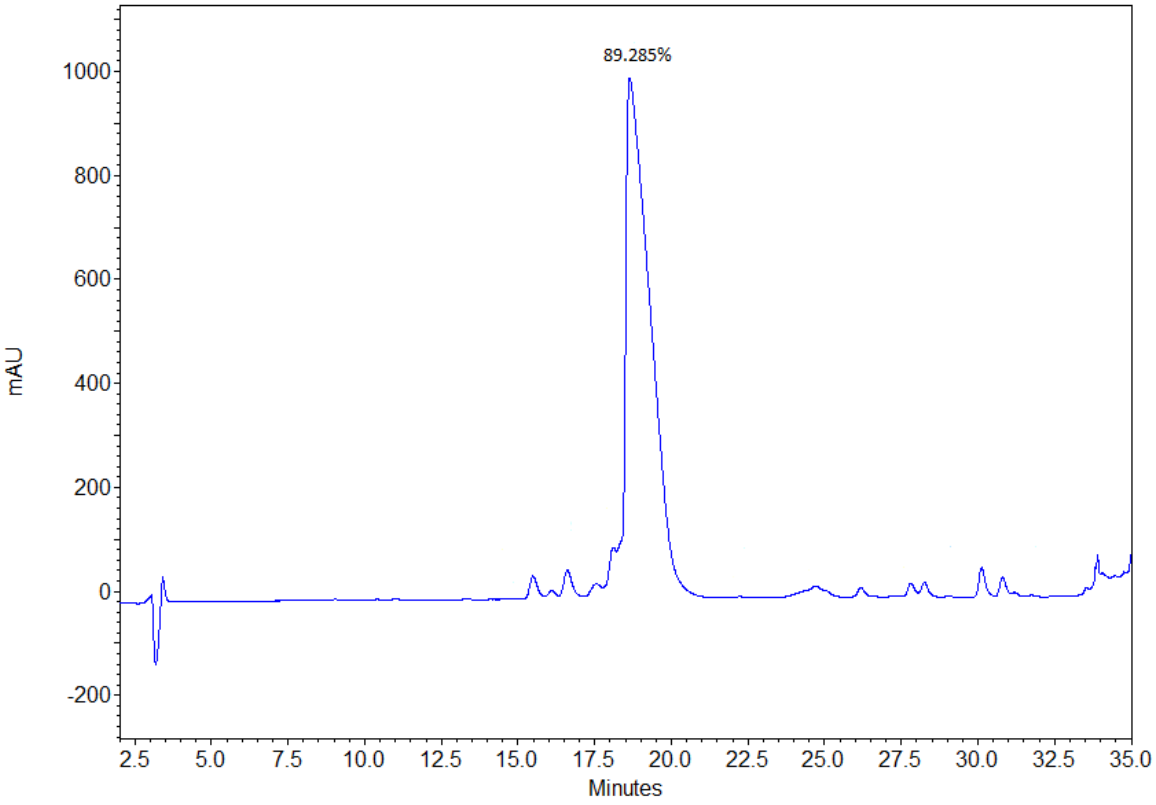


Figure 8: Reversed phase HPLC chromatogram of AoPLP peptide shows the purity of AoPLP peptide (89%).

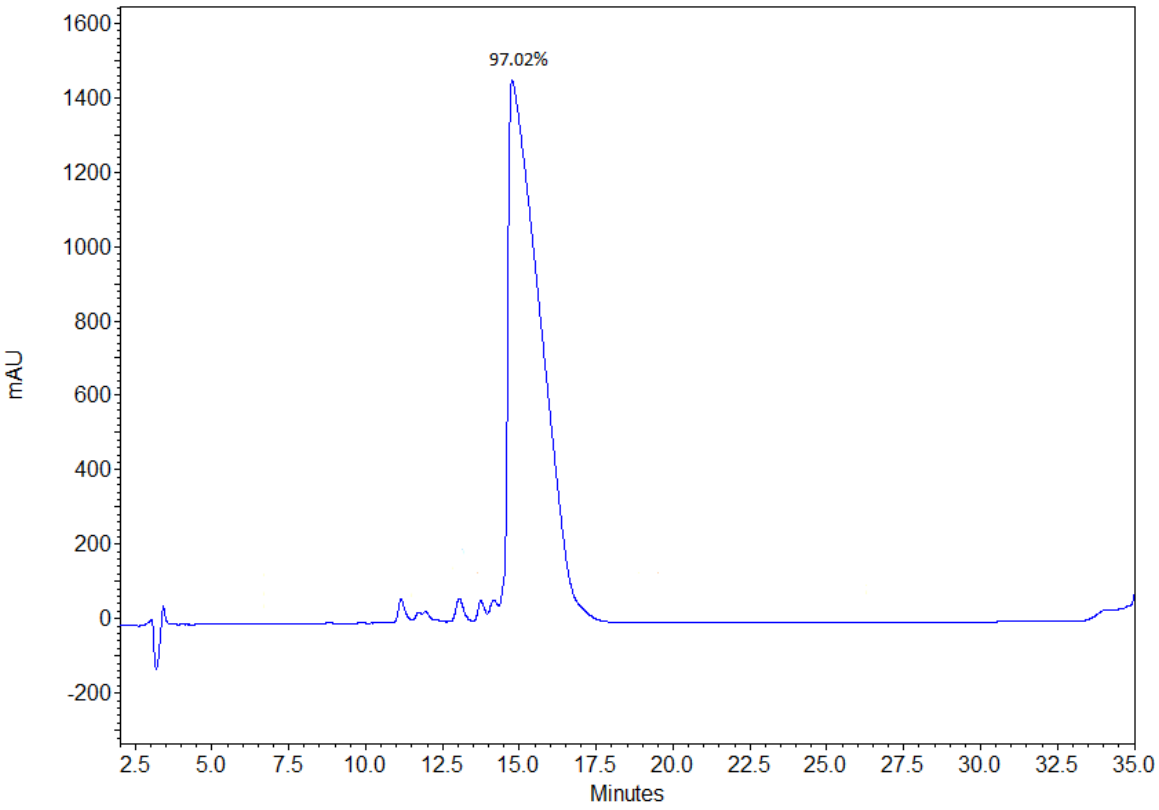


Figure 9: Reversed phase HPLC chromatogram of PLP shows the purity of AoPLP peptide (97%).

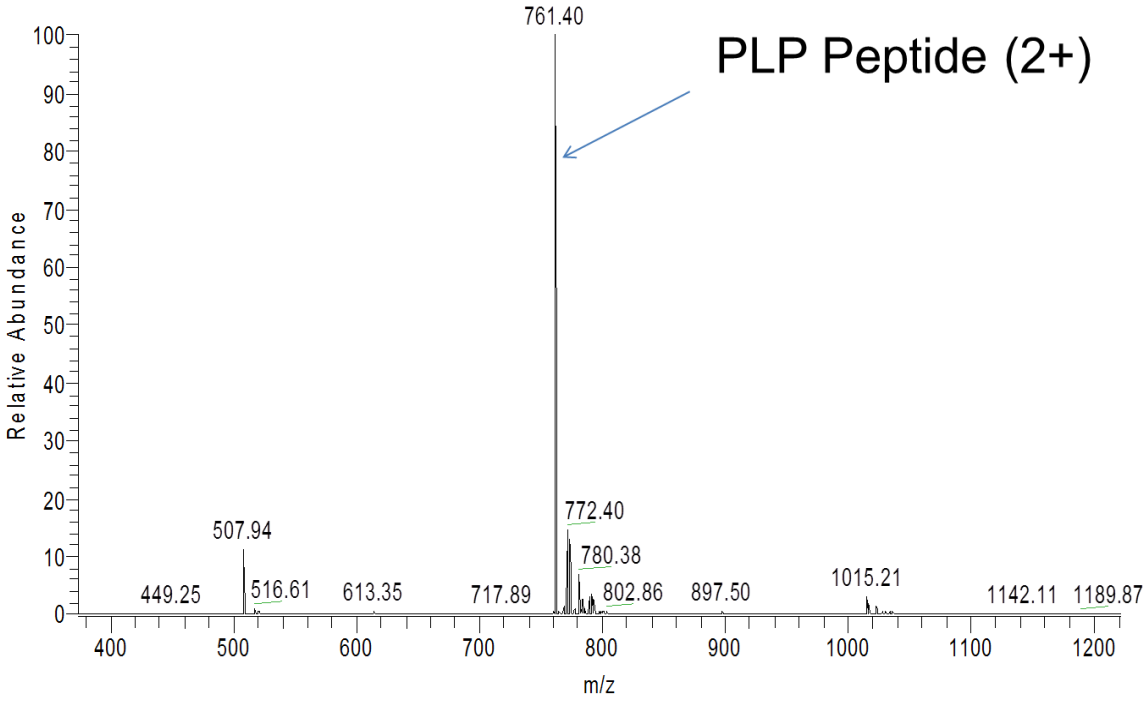


Figure 10: ESI+ mass spectroscopy of the mixture of PLP and NAG showing the absence of any product peak at pH 5, acetate buffer.

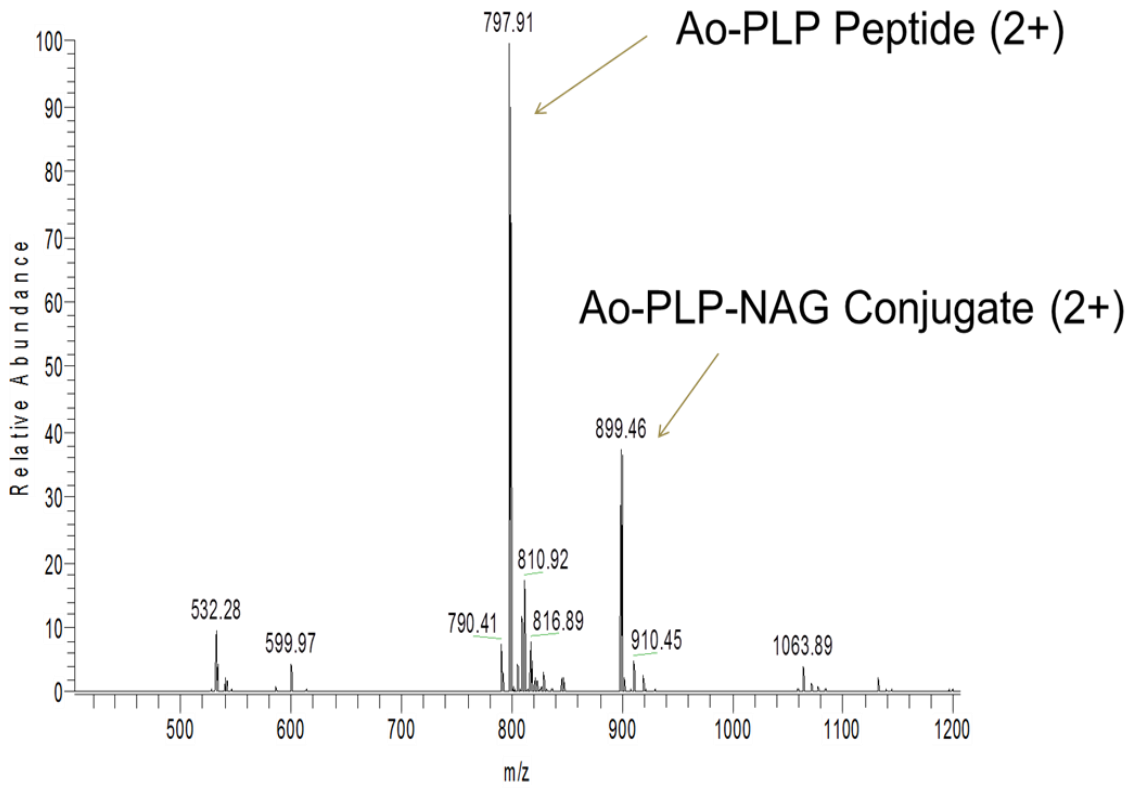


Figure 11: ESI+ mass spectroscopy of the mixture of PLP and NAG showing the presence of the product peak at pH 5, acetate buffer.

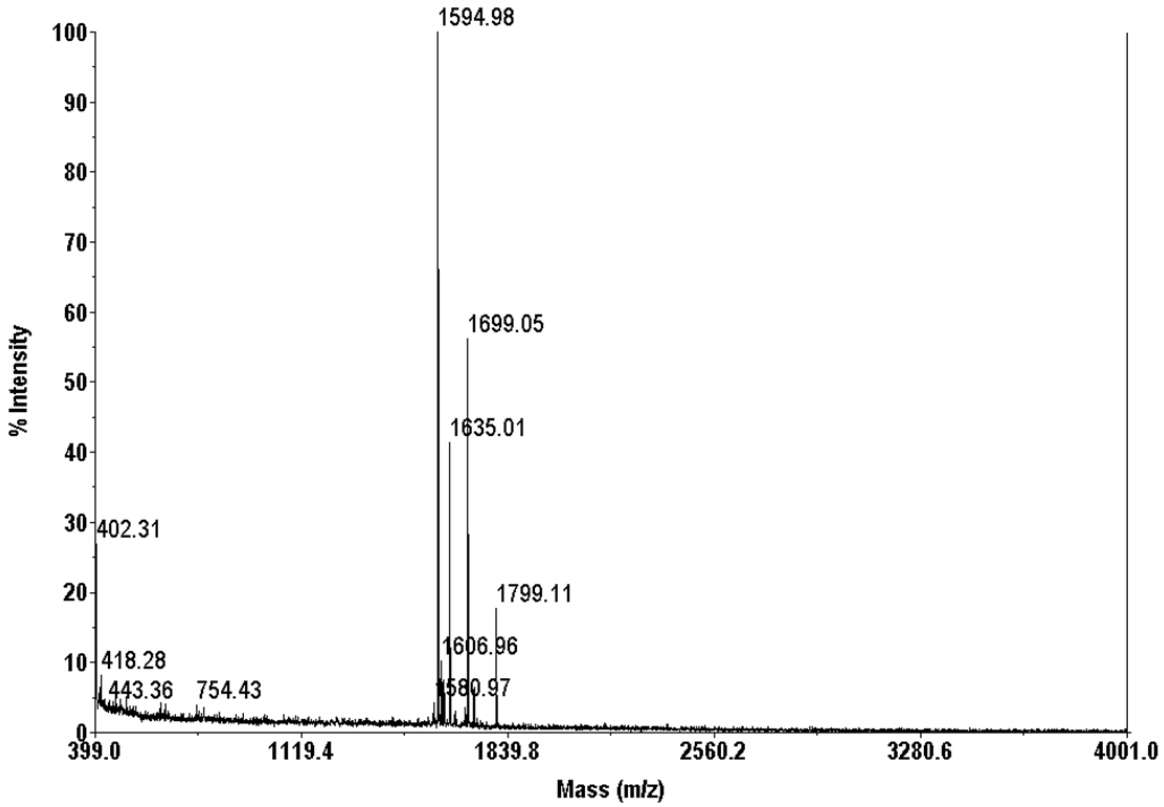


Figure 12: MALDI mass spectroscopy of the mixture of AoPLP and NAG showing the presence of the product peak at pH 6, citrate buffer.

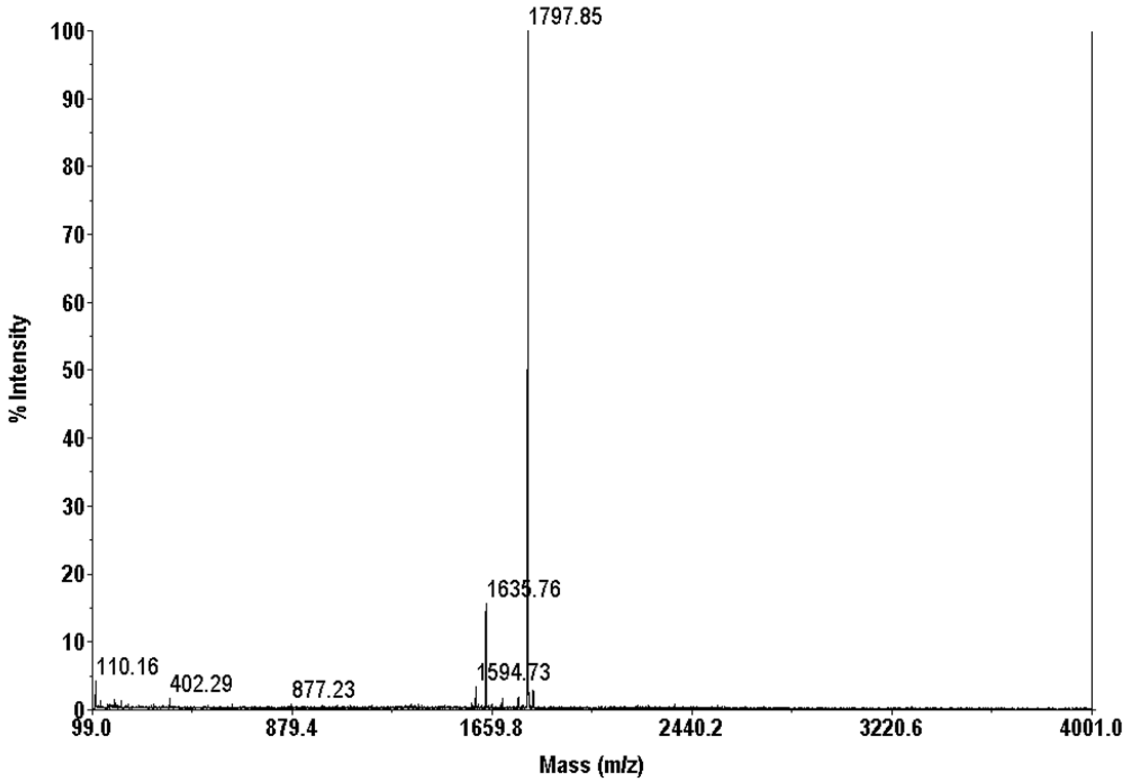


Figure 13: MALDI mass spectroscopy of the mixture of AoPLP and NAG showing the presence of the product peak at pH 7, phosphate buffer.

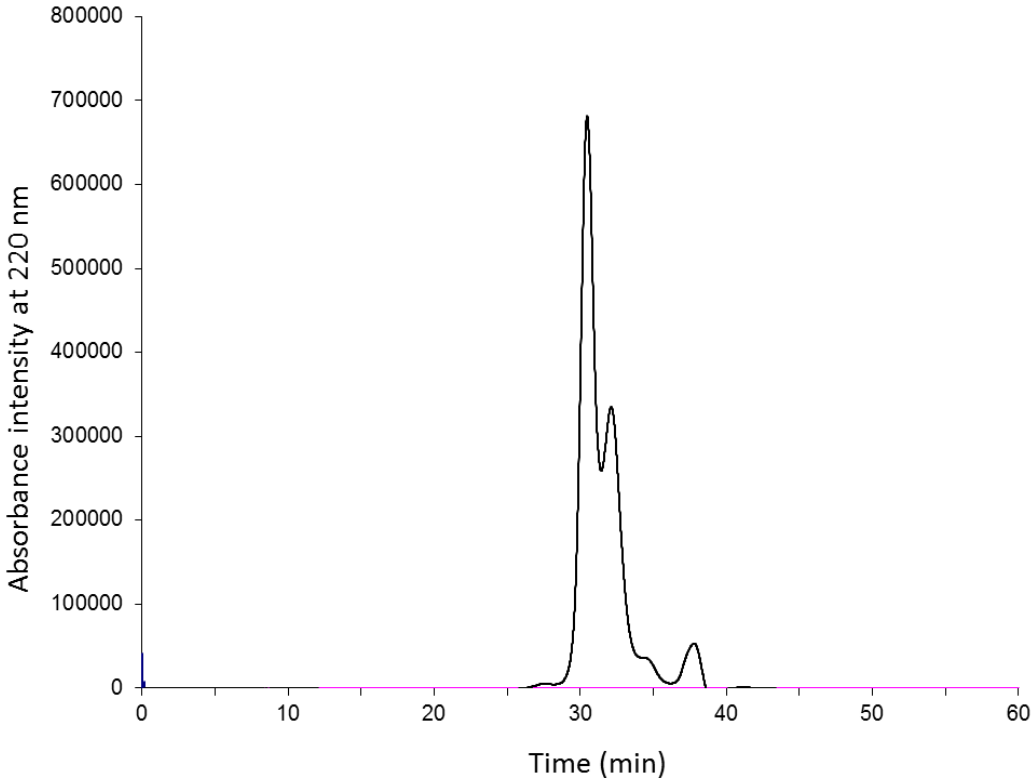


Figure 14: Size exclusion chromatogram for the mixture of AoPLP and NAG showing poorly separated peaks when 0.1 mM ammonium acetate was used as the mobile phase.

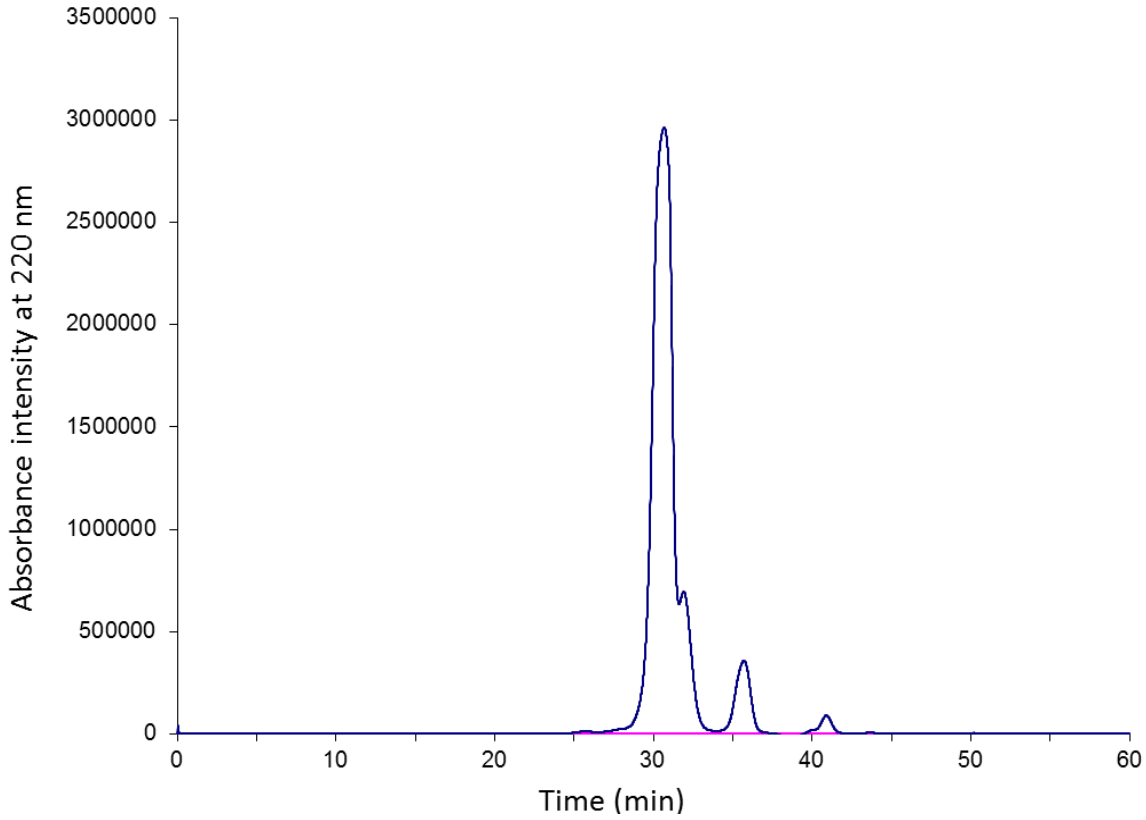


Figure 15: Size exclusion chromatogram for the mixture of AoPLP and NAG showing poorly separated peaks when acetonitrile-water (10:90) with 0.1% trifluoroacetic acid was used as the mobile phase.

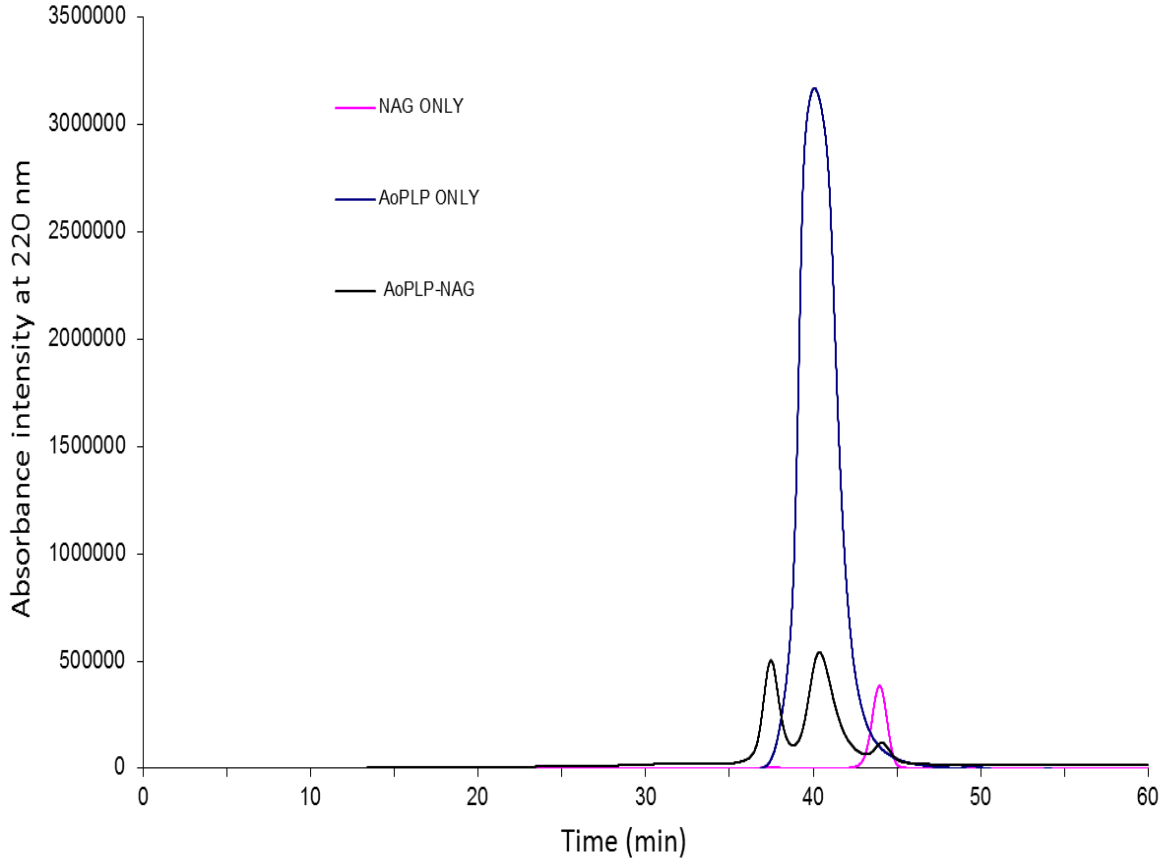


Figure 16: Size exclusion chromatograms for NAG only, AoPLP only and the product of AoPLP and NAG when 0.1 mM sodium phosphate was used as the mobile phase.

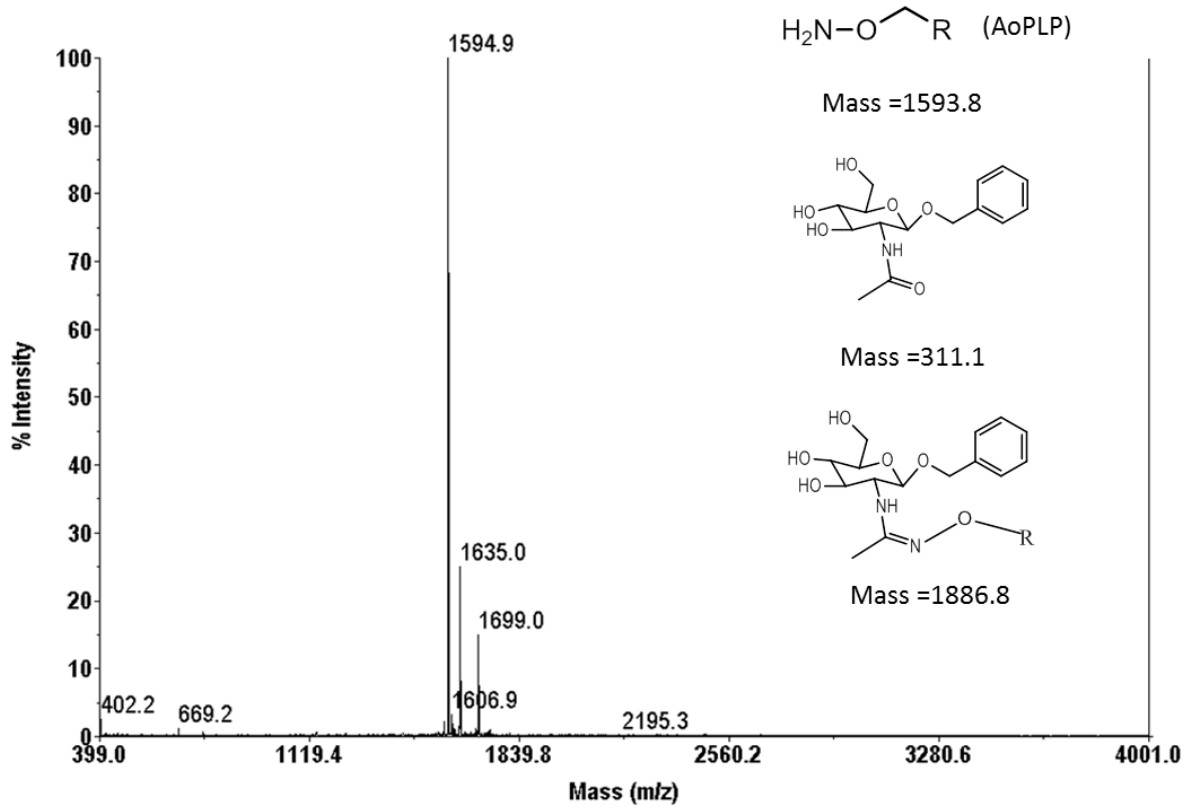


Figure 17: MALDI mass spectroscopy of the mixture of AoPLP and benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside showing the absence of the product peak (expected product mass 1886.8).

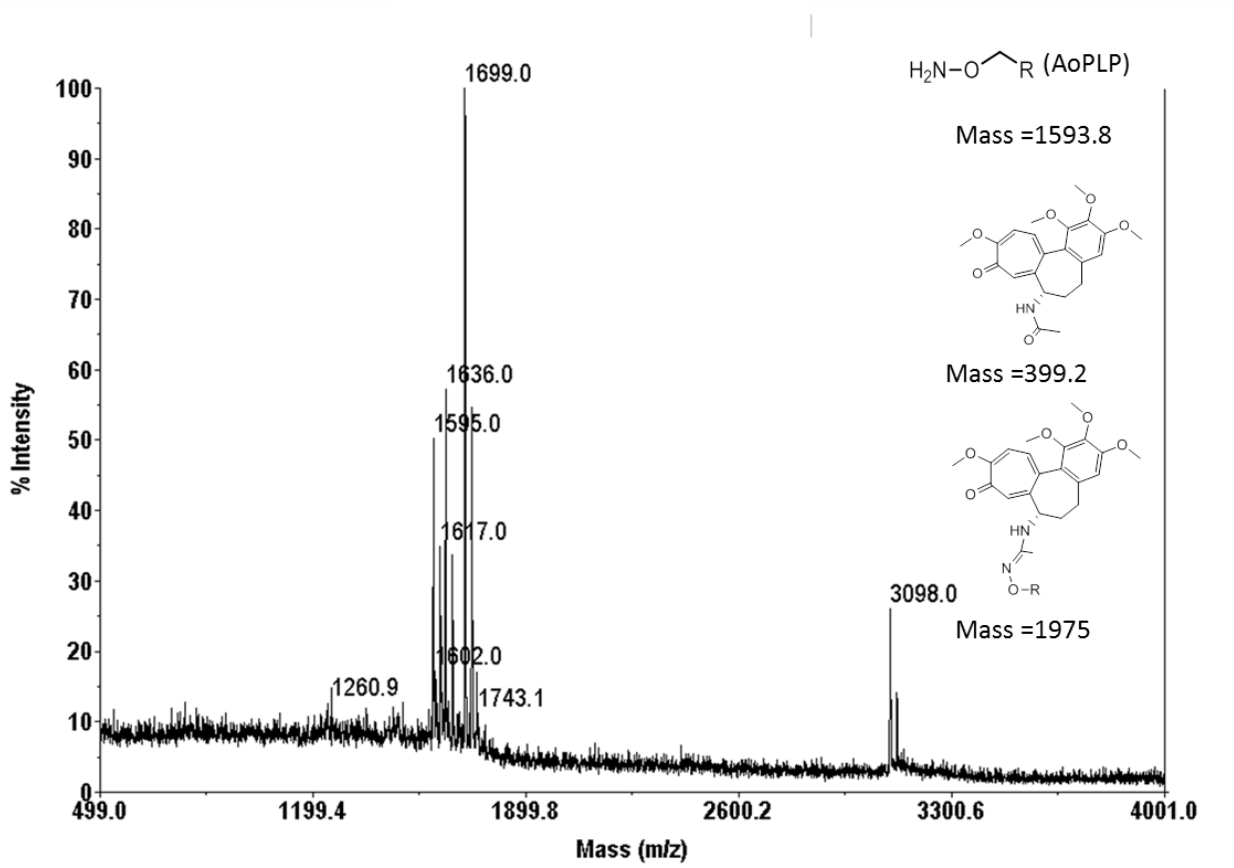


Figure 18: MALDI mass spectroscopy of the mixture of AoPLP and colchicine showing the absence of the product peak (expected product mass 1975).

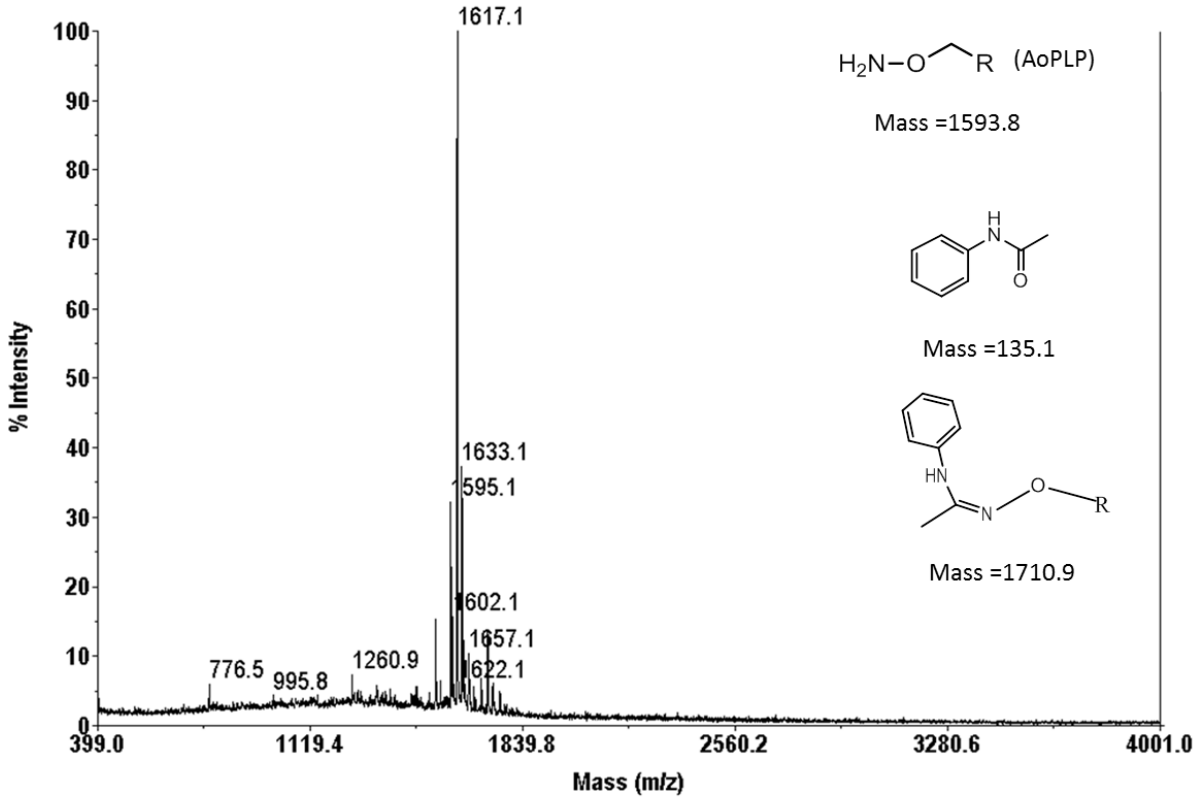


Figure 19: MALDI mass spectroscopy of the mixture of AoPLP and Acetanilide showing the the absence of the product peak (expected product mass 1711.92)

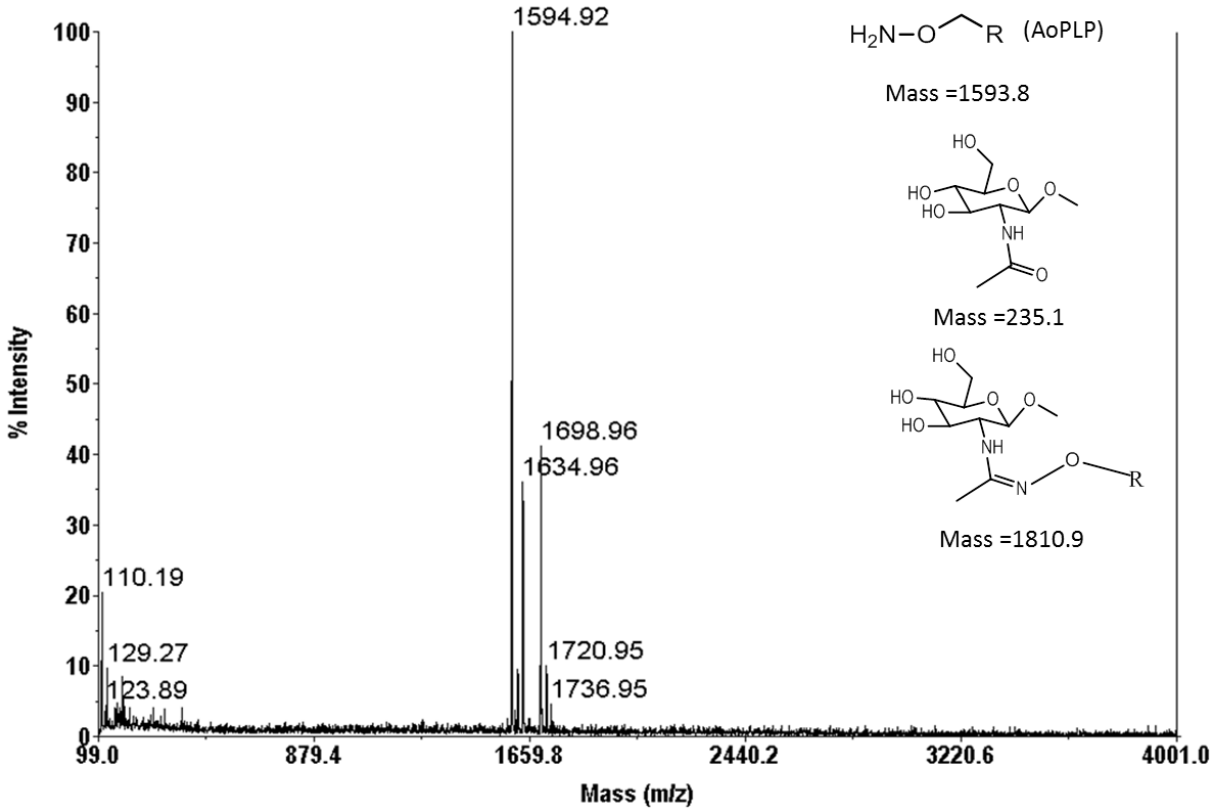


Figure 20: MALDI mass spectroscopy of the mixture of AoPLP and 3-O-methyl-N-acetyl-D-glucosamine showing the absence of the product peak (expected product mass 1810.9).

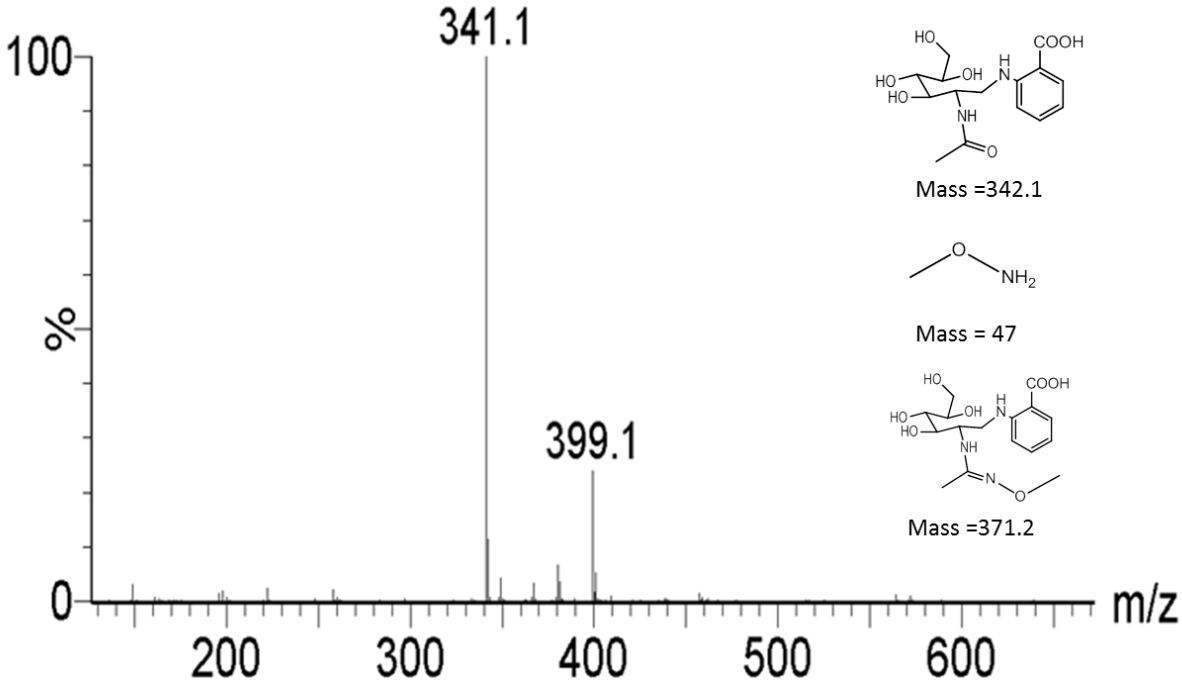


Figure 21: ESI- mass spectroscopy of the mixture of *O*-methylhydroxylamine and blocked NAG showing the absence of the product peak (expected product mass 371.2).

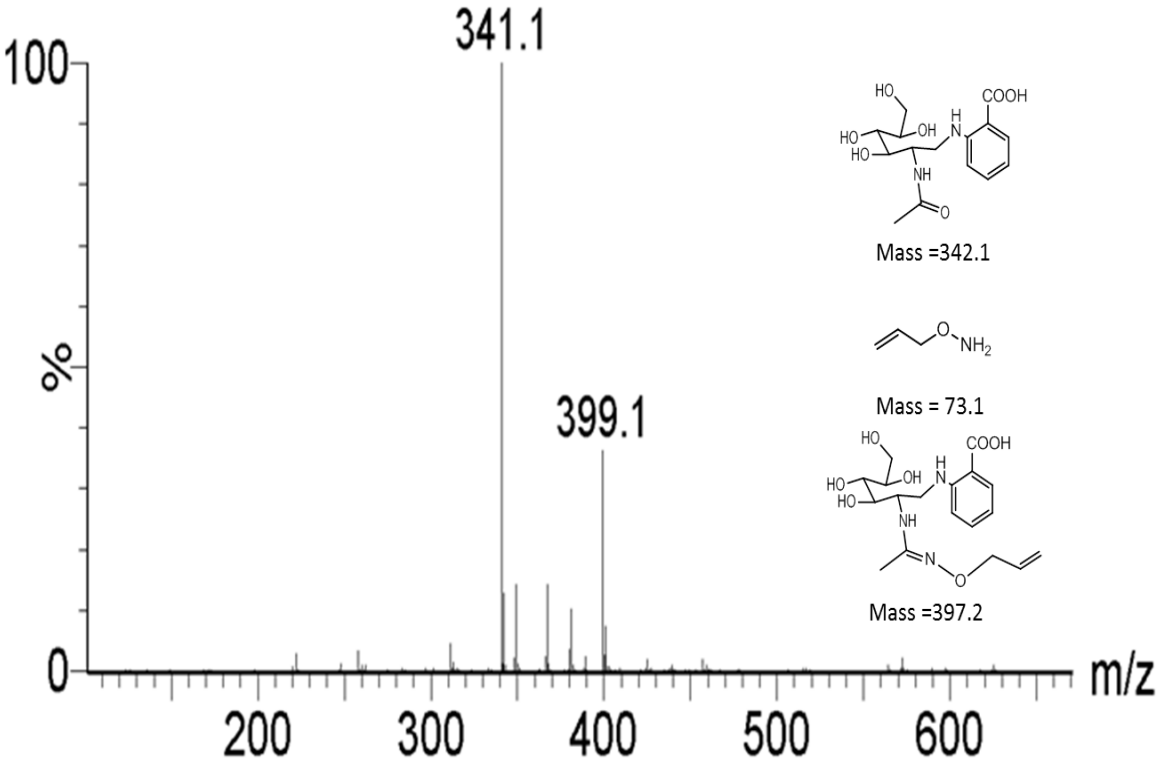


Figure 22: ESI- mass spectroscopy of the mixture of *O*-allyl-hydroxylamine and blocked NAG showing the absence of the product peak (expected product mass 397.2).

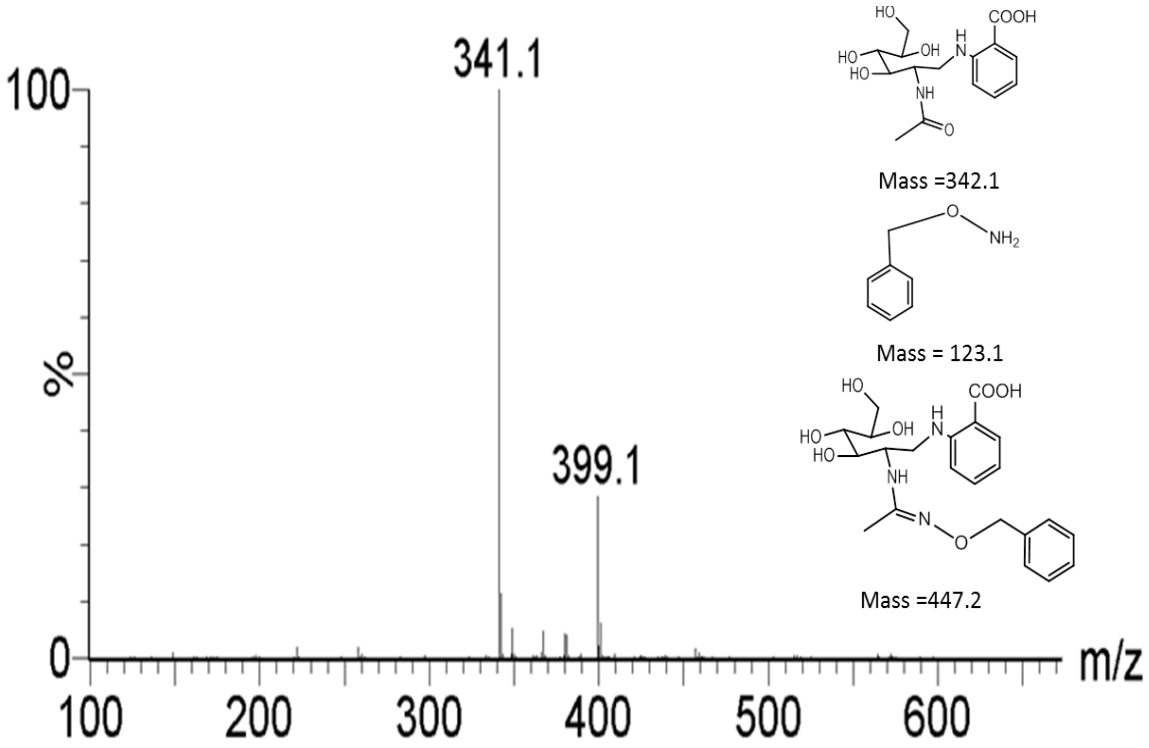


Figure 23: ESI- mass spectroscopy of the mixture of *O*-benzylhydroxylamine and blocked NAG showing the absence of the product peak (expected product Mw 447.2006).

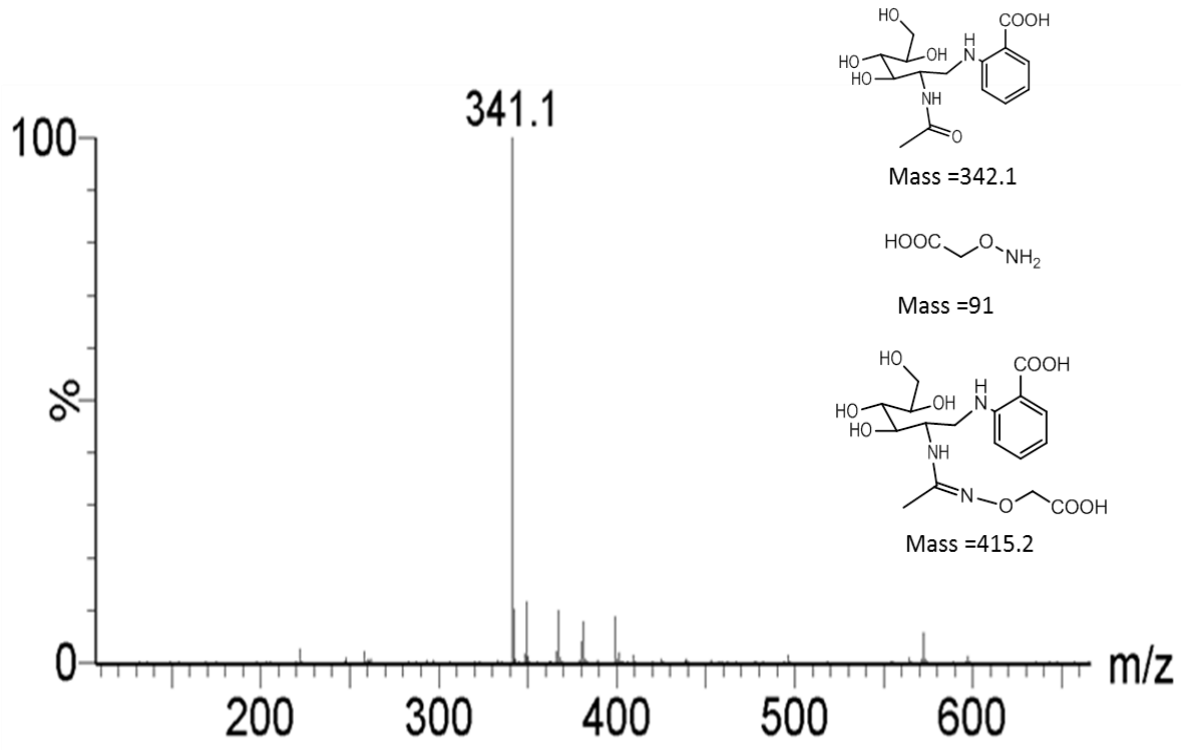
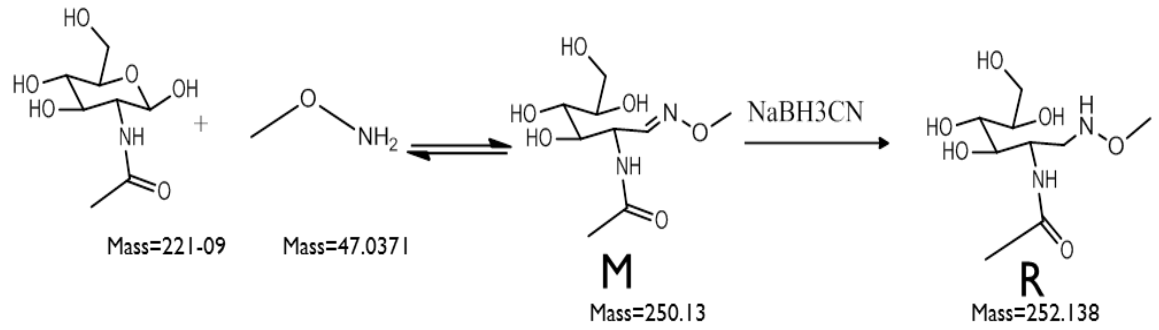


Figure 24: ESI- mass spectroscopy of the mixture O-(carboxymethyl) hydroxylamine and blocked NAG showing the absence of the product peak (expected product mass 415.2).



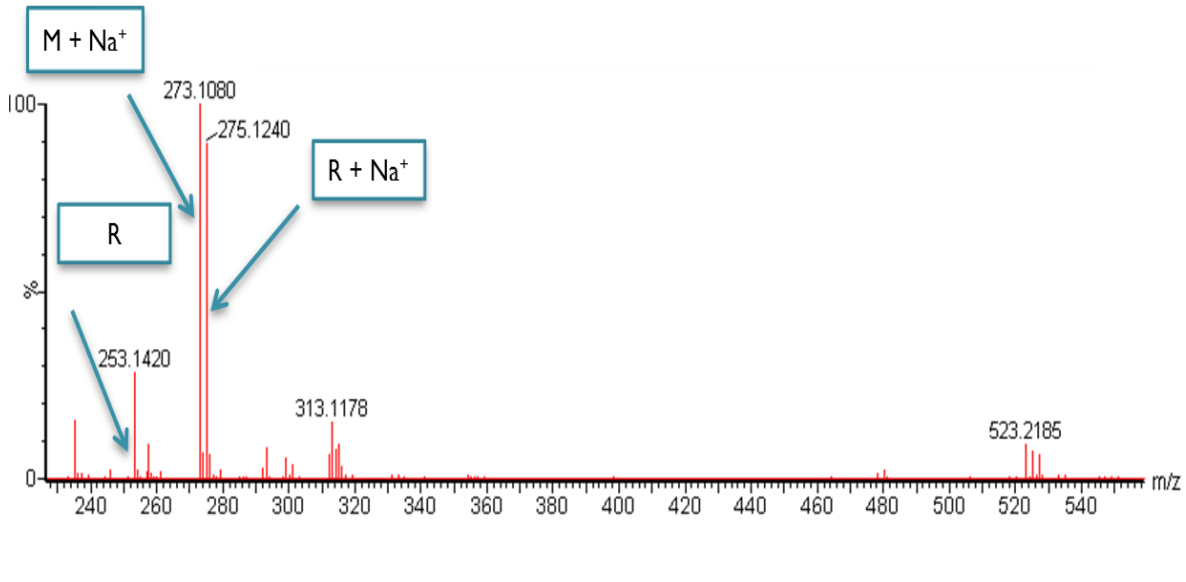
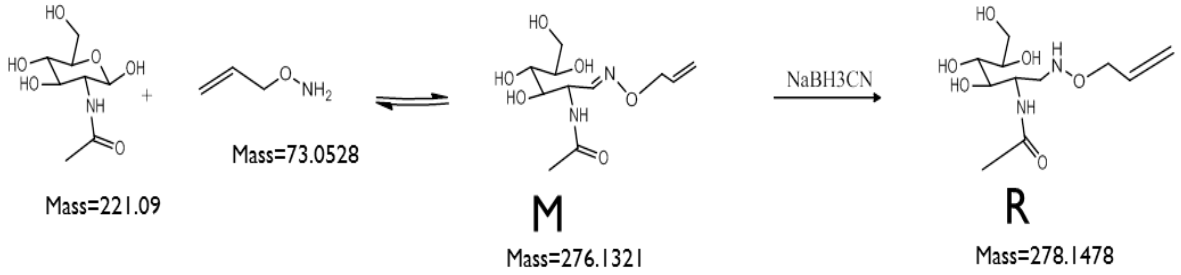


Figure 25: ESI+ mass spectroscopy of the mixture of *O*-methylhydroxylamine and unblocked NAG showing the presence of the product peak.



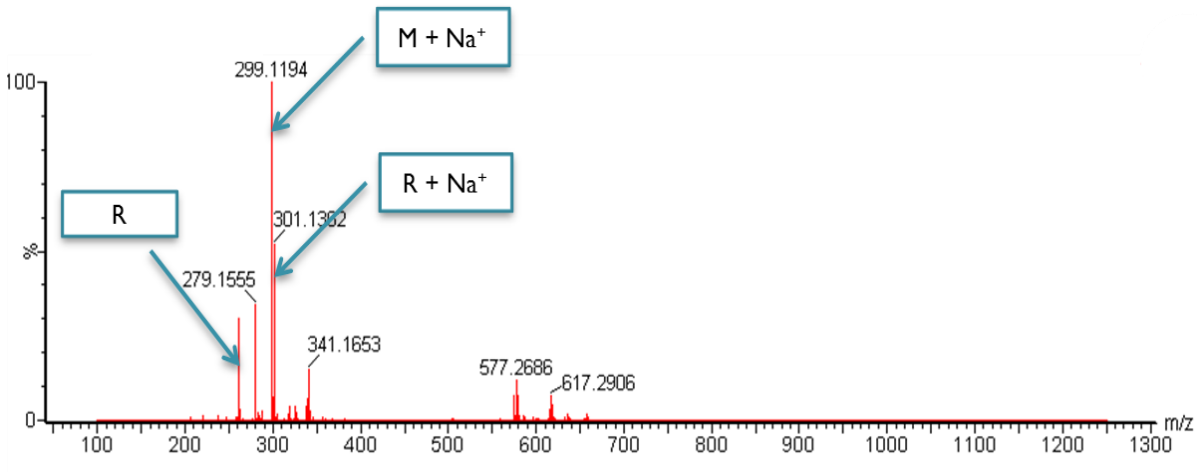
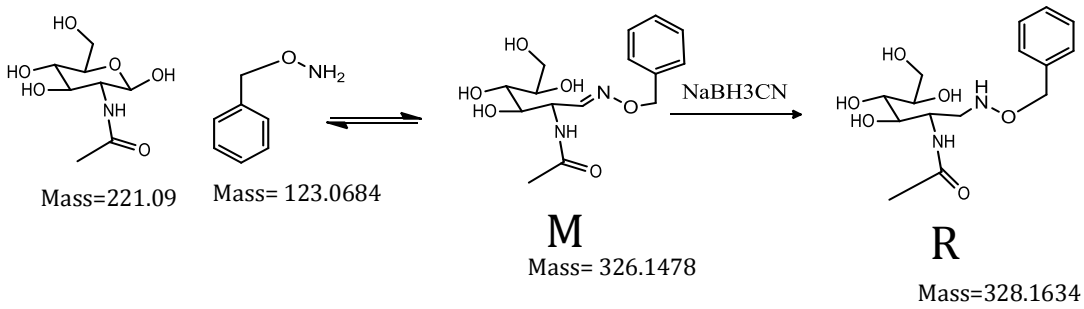


Figure 26: ESI+ mass spectroscopy of the mixture of O-allyl-hydroxylamine and unblocked NAG showing the presence of the product peak.



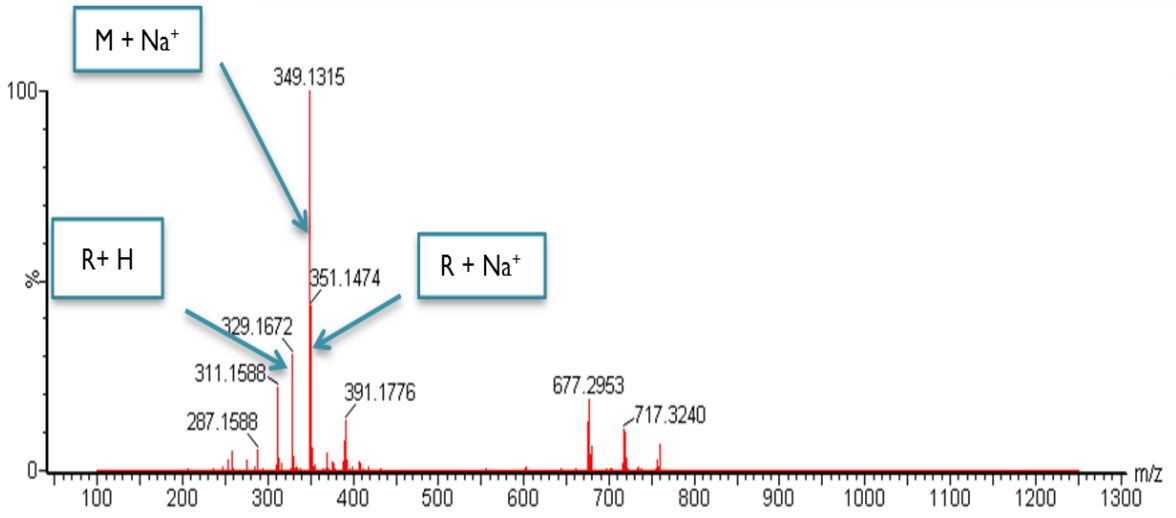
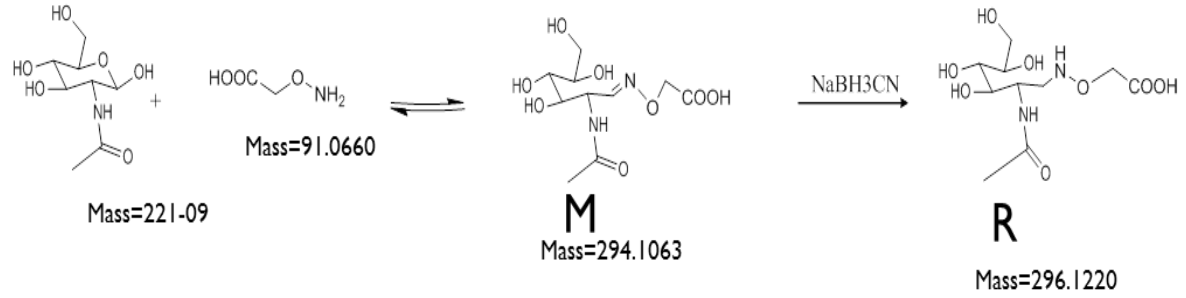


Figure 27: ESI+ mass spectroscopy of the mixture of *O*-benzylhydroxylamine and unblocked NAG showing the presence of the product peak



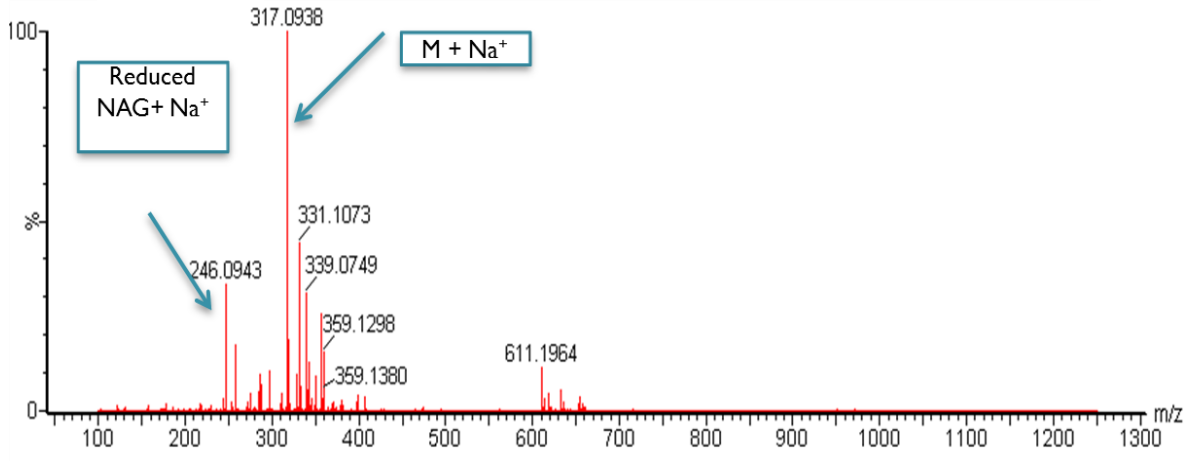


Figure 28: ESI+ mass spectroscopy of the mixture O-(carboxymethyl) hydroxylamine and unblocked NAG showing the presence of the product peak.

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