Development of Newborn Screening Methods for Mucopolysaccharidosis III type A and type B in Dried Blood Spot using Tandem Mass Spectrometry

Fan Yi

A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2018

Reading Committee:

Michael H. Gelb, Chair

Jesse Zalatan

Alshakim Nelson

Program Authorized to Offer Degree:

Chemistry

Abstract

Development of newborn screening methods for
mucopolysaccharidosis III type A and type B
in dried blood spot using tandem Mass Spectrometry

Fan Yi

Chair of the Supervisory Committee:
Professor Michael H. Gelb
Department of Chemistry

Chapter I. Development of Newborn Screening Method for Mucopolysaccharidosis III Type A in Dried Blood Spot using Tandem Mass Spectrometry

Mucopolysaccharidosis III type A (MPS III A) is an autosomal recessive inherited disease with no current treatment available. It is caused by the deficiency of sulfamidase (SGSH) in the lysosome and the inability to degrade a polysaccharide called heparan sulfate. Gene therapy and enzyme replacement therapy for MPS III A are already in development and have shown promising preclinical result. To achieve the best result from the potential treatment, it is necessary to administrate the treatment before the onset of irreversible damage to the central nervous system. To fulfill this requirement, a simple, fast and robust method for screening MPS III A in newborns is in demand. The first high-throughput screening method for MPS III A that

is compatible with dried blood spot (DBS) was developed. This method used a synthetic substrate to assay the enzyme activity in dried blood spots via tandem mass spectrometry (MS/MS). Preliminary data suggested that the newly developed assay was able to distinguish patients from normal population in sulfamidase activity: patient SGSH activity ranged from 0.013 – 0.056 μmol/h/L; while normal newborn SGSH activity ranged from 0.10 - 0.81 μmol/h/L.

Chapter II. Development of Newborn Screening Method for Mucopolysaccharidosis III Type B in Dried Blood Spot using Tandem Mass Spectrometry

Mucopolysaccharidosis III type B (MPS III B) is an autosomal recessive inherited disease with no current treatment. It is caused by the deficiency of N-acetyl glucosaminidase (NAGLU) in the lysosome and the inability to degrade a polysaccharide called heparan sulfate. NAGLU is a downstream enzyme of sulfamidase, which causes MPS III A. Sulfamidase and NAGLU are working together with several other enzymes, acetyl-CoA: α-glucosaminide acetyltransferase and N-acetylglucosamine 6-sulfatase, to cleave off the nonreducing end glucosamine residue from a heparan sulfate polysaccharide molecule. The accumulation of heparan sulfate in the lysosome will cause damage to the central nervous system which in turn will result in mild somatic and severe neurological manifestation. It is crucial to identify the affected individual as soon as possible, preferably right after birth. In order to do so, a high-throughput screening method was developed, and it had shown its power in distinguish patients from the normal people. Preliminary data suggested that a random normal person has a NAGLU activity 100 times higher than the one of the affected persons.

Table of Contents

Table of Figures

ACKNOWLEDGEMENTS

I would like to thank my advisor Michael H. Gelb for his constant support and guidance; my committee members for their advice and encouragement; and the University of Washington Chemistry Department and the National Institutes of Health for financial support throughout graduate school. My research would not have been possible without the help of numerous lab members, peers, and personnel from the Department of Chemistry.

Introductions

Lysosomal Storage Diseases

Lysosomal storage diseases (LSDs) are disorders that are caused by defects in the proteins involved in the metabolism of macromolecules in the lysosomal system. As of today, there are more than fifty identified LSDs in human. The deficiency in the enzymes, transporter proteins or the proteins responsible for post-translational modification cause the accumulation of metabolites in the lysosome, which leads to progressive cell damage and various symptoms, including skeletal abnormalities, visceromegaly and central nervous system dysfunction.[1] Most of the time, the manifestations of LSDs in the infants are not apparent and specific in the early stage of infancy, which in turn causes delayed diagnosis of LSDs. Currently, enzyme replacement therapy (ERT), gene therapy, and bone marrow transplantation (BMT) have been developed for some LSDs, such as Gaucher, Fabry and Krabbe diseases.[2,3] Some damages caused by LSDs can be partially reversed by appropriate treatment but most are not; however, studies have shown that early treatment can at least alleviate most of somatic symptoms.[4] To achieve the best outcome of the developed treatment, an early detection of the lysosomal storage diseases is necessary. All these characteristics make LSDs good candidates to be included into the Newborn Screening Project (NBS), which is an official program dedicate to identify conditions that can affect a child's long-term health or survival and enable children to reach their full potential with early detection, diagnosis, and intervention.[1]

Mucopolysaccharidosis

Mucopolysaccharidosis (MPS) is a major subfamily of LSDs; it is associated with the inability to break down glycosaminoglycans (GAGs). There are seven major subtypes of MPS currently been discovered; they are named in order of their discovery from number I to VII.

MPS III, Sanfilippo syndrome, is a collective name for four disorders: MPS III type A, B, C, and D. Four different enzymes are responsible for the four subtypes of MPS III: heparan N-sulfatase (sulfamidase, SGSH), α-N-acetylglucosaminidase (NAGLU), Acetyl-CoA: α-glucosaminide acetyltransferase (HGNAT), and N-acetylglucosamine 6-sulfatase (GNS), respectively.[5] These four enzymes are specific for degrading a GAG called heparan sulfate. Heparan sulfate is consisted of repeating disaccharides units of uronic acid and D-glucosamine with various degree of sulfation and acetylation. The uronic acid may be either α-L-iduronic acid or β-D-glucuronic acid; D-glucosamine may be either N-sulfated or N-acetylated.[6] Heparan N-sulfatase cleaves off the N-sulfate group and leave a free amine behind to undergo acetylation in order for the enzyme N-acetyl-glucosaminidase, the enzyme that is responsible for MPS III B, to cleave off the glucosamine unit from the polysaccharide chain. The accumulation of heparan sulfate leads to profound mental deterioration, hyperactivity, and mild somatic manifestation.[7] Since the ultimate result from lacking any one of the MPS III enzymes leads to the accumulation of heparan sulfate, all four Sanfilippo syndromes have similar symptoms. Therefore, different methods must be established in order to identify the specific disorder for corresponding treatment.

Newborn Screening (NBS)

As Sutton described, "Newborn screening is a public health program that aims to diagnose treatable disorders in the presymptomatic period, in a cost-effective manner".[8] As one of the most successful public health program in the US and the world, NBS has saved countless babies that were affected by treatable fatal diseases. It all started in the 1960s when Dr. Robert Guthrie discovered that phenylketonuria (PKU) could be detected by a simple bacterial inhibition test before symptoms started to manifest.[9] The patients affected with PKU could have a normal life when diagnosed early and started treatment, a simple restricted diet, right after diagnosis.

The story about PKU helped to establish the important criteria for NBS: first, the diseases must be treatable; secondly, the screening method must be able to distinguish the abnormal from the normal in the presymptomatic period; finally, the cost of the screening method must be kept low enough for everybody. Since then, the NBS panel has expanded to cover various kinds of diseases including organic acid disorders, amino acid disorders, fatty acid oxidation disorders and more.[8] As technology advances, more and more diseases will be included into the screening panel. The number of disorders to be screened is hindered by limited resources and time. The development of tandem mass spectrometry has made an enormous impact on the scope of NBS.

Tandem mass spectrometry (MS/MS)

Tandem mass spectrometry (MS/MS) has been implemented successfully to assay the activity of multiple enzymes present in dried blood spots (DBS) for LSDs, both individually and multiplexed.[10,11] It offers several advantages over the traditional methods, such as fluorometric assay and immunological assay. Tandem mass spectrometric assay is multiplexable, which means multiple enzymes can be assayed simultaneously. This can substantially reduce the time and cost for a large-scale screening project. It also measures the activity of the enzyme in blood instead of the abundance of the protein, which can cause significant false negative issue since the presence of the protein doesn't necessarily mean the enzyme is functional. MS/MS is used to detect the specific fragments of the enzymatic product, which is only detectable when there is functional enzyme present in the DBS. Thus, it is the activity of the enzyme that is been assayed. Moreover, due to the nature of a synthetic substrate, there are no endogeneous contents in the blood that will respond to the MS/MS in the same way, which reduces the background substantially. An internal standard (IS) is used to calculate the enzyme activity as well as accounting for the variation caused by incomplete extraction, analytes absorption on to surfaces, product consumption by

downstream enzymes and the differential suppression of ion formation.[12] In summary, Tandem MS provides a fast, specific, and multiplexable way to conduct large-scale screening project.

Chapter I. Development of Newborn Screening Method for Mucopolysaccharidosis III Type A in Dried Blood Spot using Tandem Mass Spectrometry

1. Introduction

Mucopolysaccharidosis III type A (MPS III A) is an autosomal recessive inherited disease with mild somatic and severe neurological manifestation.[5] It is caused by the defect in an enzyme called heparan N-sulfatase (sulfamidase, SGSH, EC 3.10.1.1). Due to the lack of function of sulfamidase, MPS III A patients are not able to degrade a glycosaminoglycan named heparan sulfate. Infants who are affected by MPS III A usually will not live through the second decade of their lives.[13] There is no current treatment for MPS III A; however, intra-cerebrospinal fluid gene therapy and intrathecal enzyme replacement therapy have shown promising early clinical results.[14,15] With future treatment in mind, MPS III A patients are still facing yet another problem: the diagnosis of this disorder. Because of the fast progression of MPS III A, the damage to the nervous system the patients sustained by the time the diagnosis was made (usually, when the symptom started to manifest) is already beyond repair. This necessitates an early diagnosis for MPS III A. The best chance for the patient to be caught soon after birth is to incorporate MPS III A into the Newborn Screening Project (NBS). However, the diagnosis of MPS III A relies on urinary GAGs analysis, enzyme assay with radiolabeled substrate,[16] and fluorescence-based assay.[17] All of those methods are either unreliable, due to the complexity of GAG structure; or requires extensive sample preparation, such as preparing leukocytes, fibroblasts.[5,18] Hence, the implementation of these methods for NBS is hindered by their nature.

Since the early 1990s, newborn screening lab began to utilize tandem mass spectrometry (MS/MS) as a fundamental instrument for screening various inborn errors.[19] This new tool has enabled the development of several enzyme activity assays in dried blood spot (DBS) by the

Gelb lab in the past decade.[11,20,21] They had also developed an enzyme activity assay for MPS III A with MS/MS in leukocyte and fibroblast.[5] However, MPS III A assay remains unpractical in DBS for unknown reasons. One important aspect about the method for NBS is that it must be highthroughput in order to accommendate the increasing number of newborns. Without a robust method that is compatible with DBS, the chance for MPS III A to make its way into the NBS panel is limited.

2. Sulfamidase and its functions

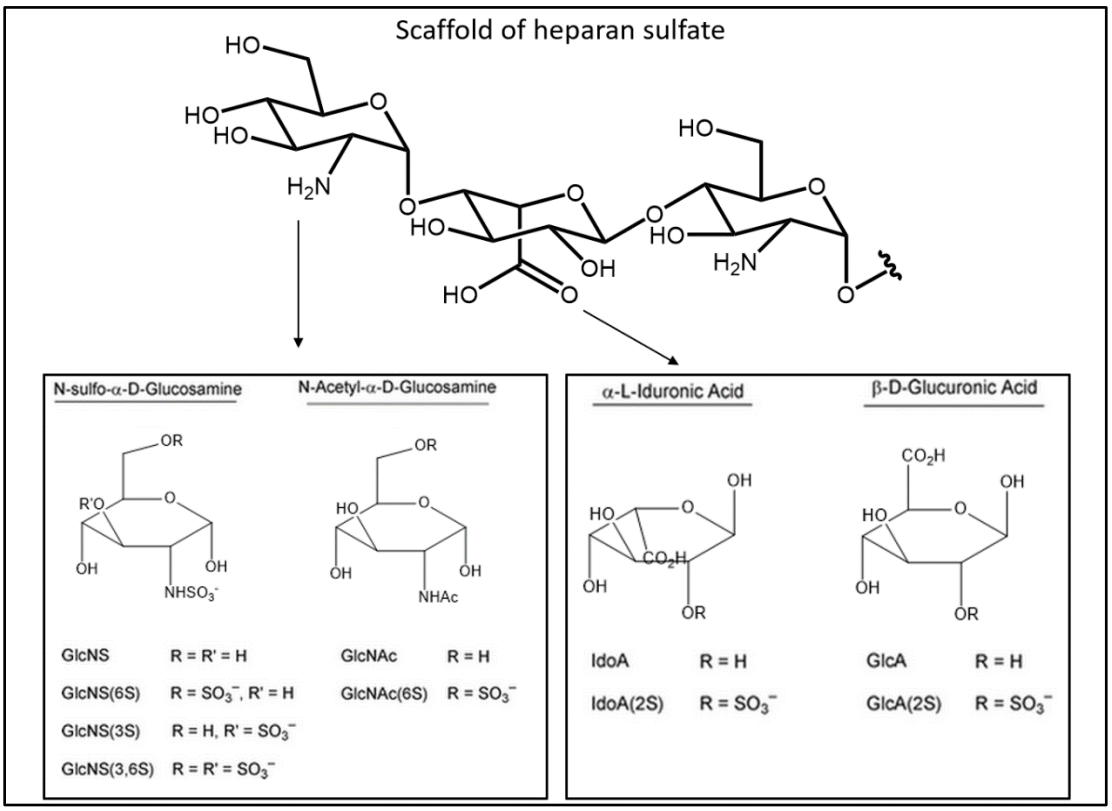


Figure 1. Heparan sulfate structure. Left bottom panel: structures for glucosamine residue; right bottom panel: structures for uronic residue.

Sulfamidase is an enzyme that catalyzes the hydrolysis of the 2-sulfate on glucosamine of a heparan sulfate. Heparan sulfate is a polysaccharide that is consisted of repeating disaccharide unit

(Figure 1 1). The disaccharide units include a glucosamine alpha 1-4 linked to a uronic acid. The uronic acid can either be an iduronic acid or a glucuronic acid. As shown in Figure 1, glucosamine unit in heparan sulfate has different substitutions on 2-, 3- and 6- position; uronic acid has a different substitution on 2- position as well. The variety of the substitution gives heparan sulfate its structural diversity and complexity, which is closely related to its biological function. Moreover, a series of enzymes are required to completely hydrolyze heparan sulfate because of this structure diversity. Sulfamidase is one of the many enzymes that are responsible for this action. Since all of the enzymes act as an exoenzyme, the whole degradation of heparan sulfate will come to a stop when one enzyme is not functioning properly.[22]

In the event of sulfamidase malfunctioning or absence, the lysosome cannot cleave the sulfate group from the 2-amino group on the glucosamine. The retention of the sulfate group causes a complete halt of heparan sulfate degradation and storing of heparan sulfate inside the lysosome, which leads to a disease named mucopolysaccharidosis III type A (MPS III A).[23] The relevance between the storing of heparan sulfate and the pathology of MPS III A is still unknown but this does not undermine the significance of this disease as it is fatal and untreatable as of today.[24]

With ongoing effort to develop a treatment for MPS III A,[15,25] a robust method for detecting this disease is also in demand. As mentioned above, the degradation of heparan sulfate requires collaboration between different enzymes. Any one of the enzymes' malfunction can cause the accumulation of heparan sulfate in cells and leads a nonspecific spectrum of symptoms. Thus, the diagnosis of MPS III A can be very difficult and the delay in diagnosis usually means the failure of a treatment if one is available. In order to specifically identify MPS III A from all other mucopolysaccharidosis (MPS), an enzymatic assay in DBS with tandem mass spectrometry

(MS/MS) was proposed, but a viable substrate for sulfamidase must be found for this method to work.

3. Development of a suitable substrate for sulfamidase

3.1. The first generation sulfamidase substrate with a mass spectrometry (MS) signal enhancer aglycone structure

3.1.1. Synthesis of the first-generation sulfamidase substrate

In a previous report by Chennamaneni[12], a novel MS signal enhancer structure was proposed. It is consisted of a di-methylene separated bis-amide moiety with various length of side chains (Figure 2A). This MS enhancer utilizes the bis-amide moiety to form a proton trap which has a high affinity for positively charged proton. In this way, the formation of the parent ion is enhanced. Upon collision-induced dissociation (CID) in the collision cell of a mass spectrometer, this signal enhancer will go through a dominant fragmentation pathway to give a major fragment (Figure 2B) which carries a positive charge and is used as the daughter ion in MS. With the newly developed MS signal enhancer as aglycone for the first design of the sulfamidase substrate, the first generation SGSH substrate was proposed as it shows in figure 3.

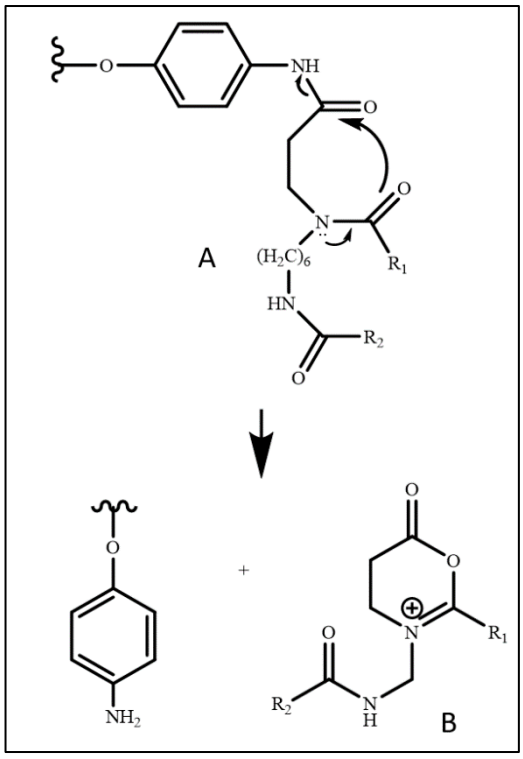


Figure 2. MS signal enhancer (A) and its pathway upon CID to the dominant fragment (B).

The synthesis of the first generation SGSH substrate was shown below. The glycosyl donor (Figure 4) and the aglycone (Figure 5) were prepared separately. Following the procedure developed by Alper and coworkers[26], compound **3** was made from D-glucosamine hydrochloride over two steps: azido group transfer and acetylation of free hydroxide groups. Compound **3** was treated with hydrazine acetate to yield compound **4**, which had a selectively deprotected anomeric hydroxide group. The free hydroxyl group was subjected to fluorination by diethylamino-sulfur trifluoride (DAST) to afford compound **5**. [27]

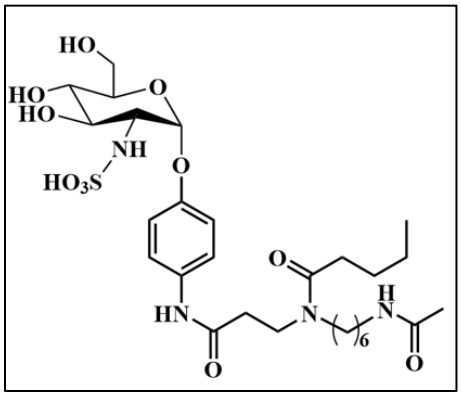


Figure 3. Structure of the first generation sulfamidase substrate.

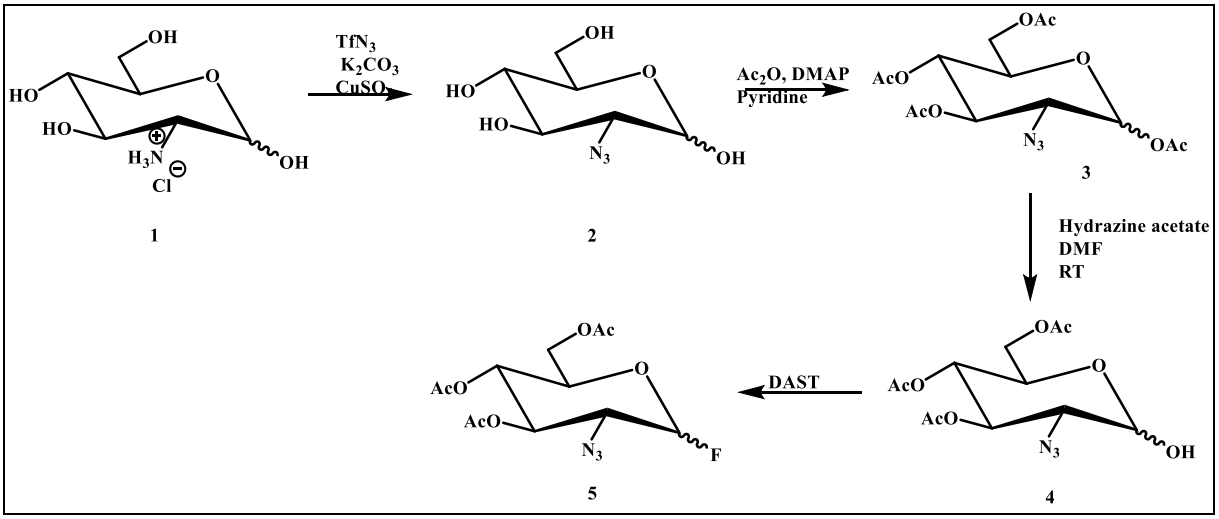


Figure 4. Synthesis of glycosyl donor for SGSH substrate.

Hexane-1,6-diamine and methyl acetate were stirred in 100 °C water for 24 hours in order to yield compound **6**. Compound **7** was prepared by a nucleophilic acyl substitution between 4-aminophenol and acryloyl chloride. The precursor of the final aglycone was yielded by a Michael addition between **6** and **7**; finally, the aglycone, compound **8**, was achieved by adding the pentanoyl moiety to the secondary amine.[5]

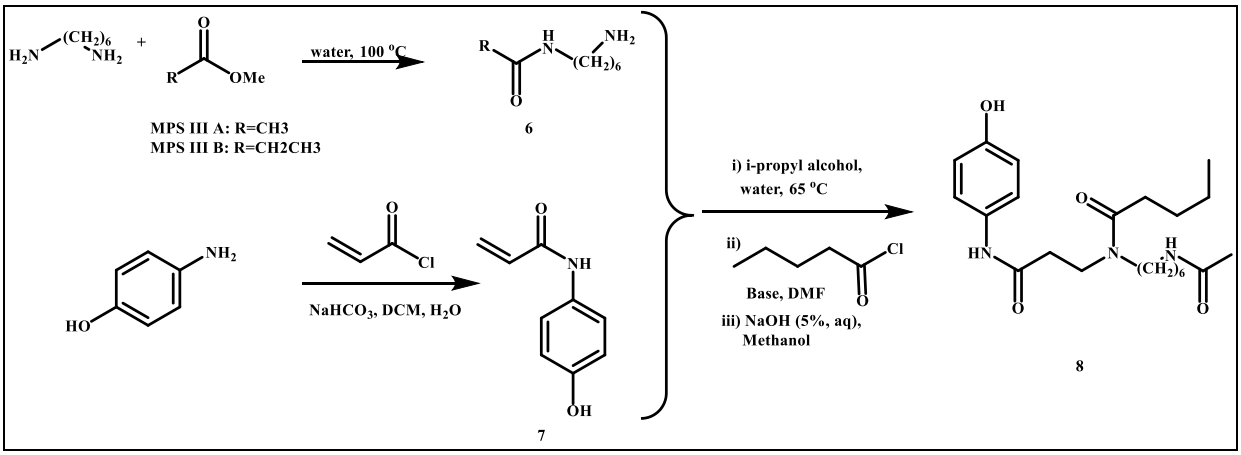


Figure 5. synthesis of the aglycone for SGSH substrate

To yield compound **9**, boron trifluoride diethyl etherate was used to conjugate the sugar moiety and aglycone.[28] Compound **10** was obtained by reducing the azido group to free amine. Until this step, all the products were mixtures containing both alpha and beta glycosides. In order to separate the alpha and beta isomers of **11**, a trifluoroacetic anhydride was used to acetylate the amine, which then allowed the separation by silica column chromatography. The final sulfamidase substrate, compound **13** was achieved after 2 steps: deacetylation and sulfation of the amine (Figure 6).[5]

3.1.2. MPS III A assay with the first generation sulfamidase substrate

With previous reports about sulfamidase assay[5,16], the initial assay buffer condition was established as follow: 50 mM sodium acetate, 7.5 mM barium (II) acetate, 5 mM cerium (III) acetate and pH 5.5. Each assay uses one 3 mm dried blood spot (DBS) punch (equivalent to 3.2 µL of blood) in a 96 deep well plate. To the DBS punch, 30 µL of buffer containing 1 mM MPS III A substrate and 1 µM MPS I internal standard (used as a tentative IS) was added and put in the 37°C orbital shaker for 16 hours at 250 rpm. The assay was then quenched by adding 120 µL of acetonitrile to each well, followed by 5 minutes of centrifugation at 3000 rpm to pellet the

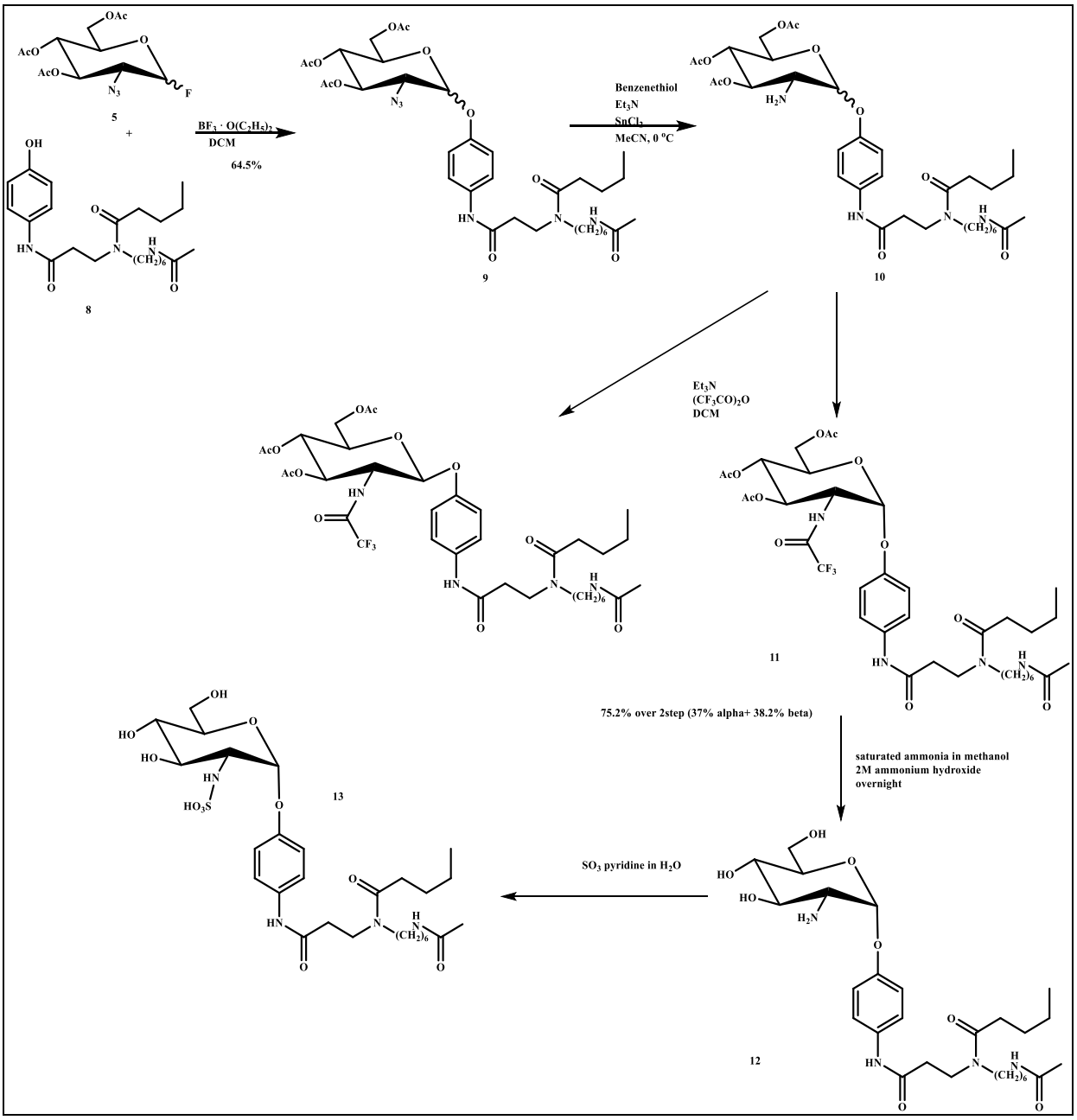


Figure 6. Synthesis of final first generation sulfamidase substrate

precipitate. 120 µL of the supernatant was then transferred to a shallow well plate and 120µL water was added to the plate. Then the plate was sealed with aluminum foil and subjected to LC/MS/MS directly. Mass spectrometry was performed on a Waters Xevo instrument using positive ion mode selected reaction monitoring (SRM) with tuned parameters that optimize the

signal from the enzymatic product. An assay was performed using quality control (QC) blood obtained from the Center for Disease Control (CDC)[29] to confirm the activity of the DBS sulfamidase. QC blood was prepared with mixing healthy pooled blood and heat deactivated blood, which is considered as a base pool blood with no residual enzyme activity. Quality control base pool (QC BP), low (QC L), medium (QC M) and high (QC H) are prepared with 0%, 5%, 50% and 100% healthy blood, respectively. The result was shown in Figure 7, QC DBS were used along with blood from a random normal person. Data shows no differentiation among all the different types of DBS despite the fact that the different types of DBS contain different amount of sulfamidase. This suggested two things: first, compound **13** is not a substrate for sulfamidase; secondly, it is not a suitable substrate for sulfamidase in DBS because of a low V_{max} and low concentration of sulfamidase present in DBS.

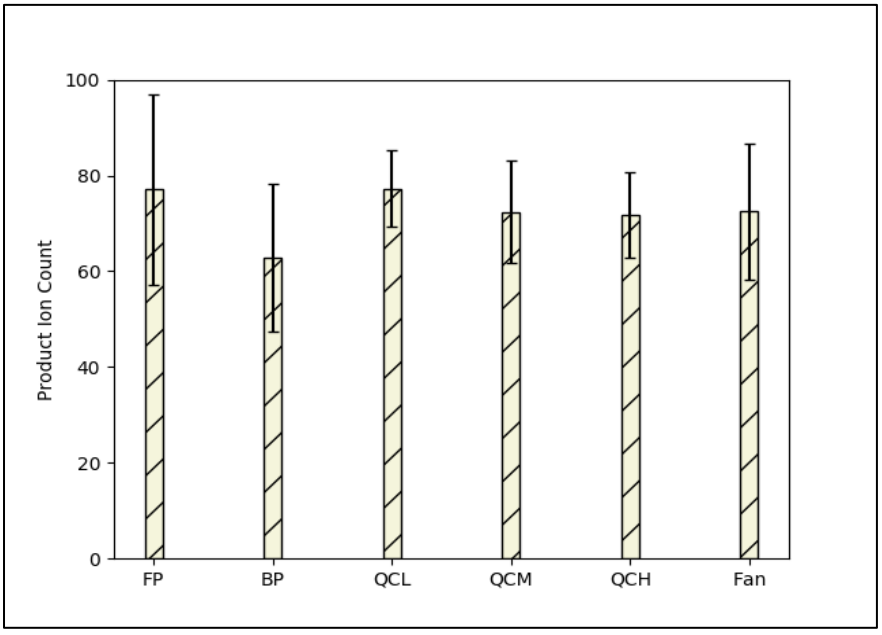


Figure 7. Product ion count in various DBS samples. FP: filter paper; BP: base pool; QCL: quality control low; QCM: quality control medium; QCH: quality control high; Fan: normal blood. Since no internal standard has been made yet, enzyme activity is reported in the format of product ion count.

To address the first possibility, recombinant sulfamidase was purchased from R&D research (cat # 8380-SU-020). A standard curve was made to demonstrate that compound **13** could be recognized by rhSGSH and there was a positive correlation between the concentration of rhSGSH used in the assay and the amount of product formed.

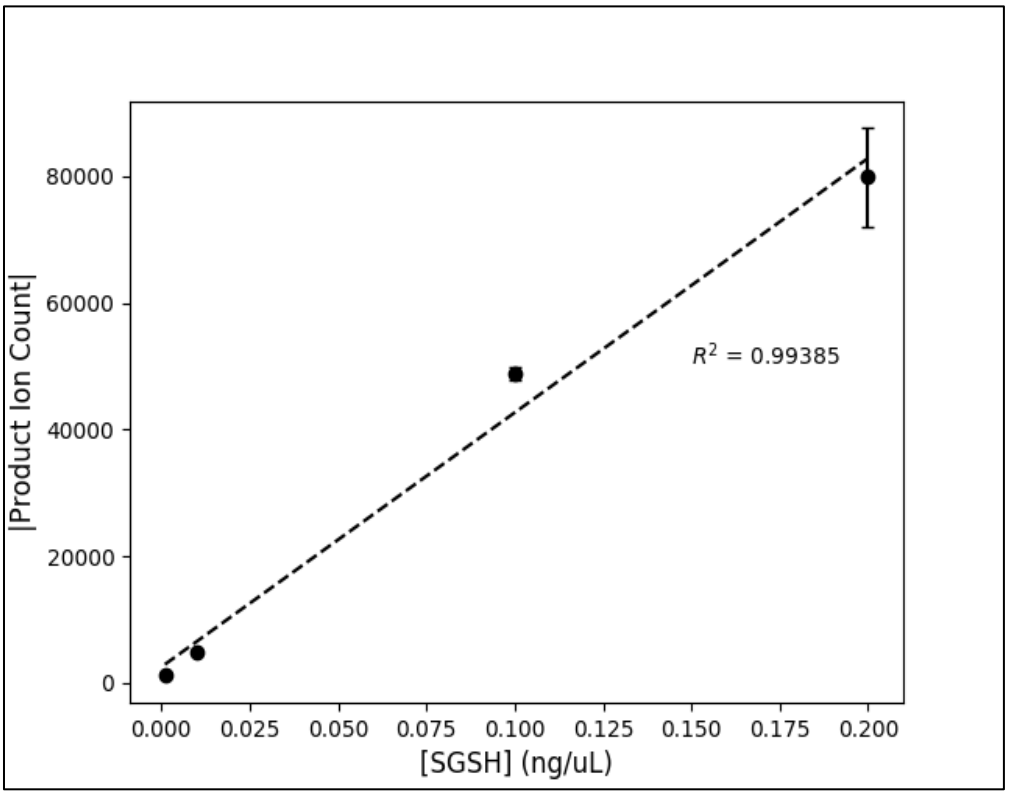


Figure 8. Correlation between product ion count and the concentrations of recombinant human sulfamidase in assay. SGSH was stored in -80 °C and thaw on ice before addition to buffer.

As shown in Figure 8, the amount of product formed by sulfamidase action increases proportionally with the concentration of rhSGSH used in the assay, which indicates that compound **13** is a suitable substrate for sulfamidase; however, it does not provide a sufficient V_{max} for this enzymatic reaction. Before a conclusion of the usefulness of compound **13** as a sulfamidase substrate in DBS is reached, further investigation of assay conditions was

conducted. Several reports on MPS III A diagnosis have stated that both sulfate and phosphate are potent inhibitors for sulfamidase.[16,18] In DBS, both sulfate and phosphate are present endogenously. To circumvent this problem, different amount of barium and cerium ion was introduced into the assay as a buffer salt, to precipitate the sulfate and phosphate ion, respectively. However, no observable product can be detected with all those conditions. Furthermore, according to Karpova and coworkers,[18] the presence of a protease inhibitor, pefabloc, could provide a 3-4 fold increase in sulfamidase activity. They claimed that the protease inhibitor could prevent any possible proteolysis of sulfamidase when it came into contact with any protease. Unfortunately, it had been proven false in a DBS assay with compound **13.** Afterall, Karpova's finding is from an sulfamindase assay in fibroblast and leukocyte, which are not the same as DBS. DBS, as a media for NBS sample transportation, exhibits different properties than others since it is a more complex system.

3.2. The second generation sulfamidase substrate with a mass spectrometry (MS) signal enhancer aglycone structure and a disaccharide moiety

3.2.1. Synthesis of the second-generation sulfamidase substrate

The failure of the first generation sulfamidase substrate leads to a conclusion that a substrate with a better V_{max} must be found. With a study conducted by Hopwood and coworkers, they discovered that the extension of one more saccharide unit in the substrate increases the catalytic efficiency of sulfamidase dramatically.[30] In this study, they have shown a disaccharide substrate had a catalytic efficiency 359 times higher than a monosaccharide substrate, which pointed the direction for the design of a better substrate. Disaccharide substrates were made as shown in figure 9 to figure 12. Starting from the building blocks essential for the disaccharide.

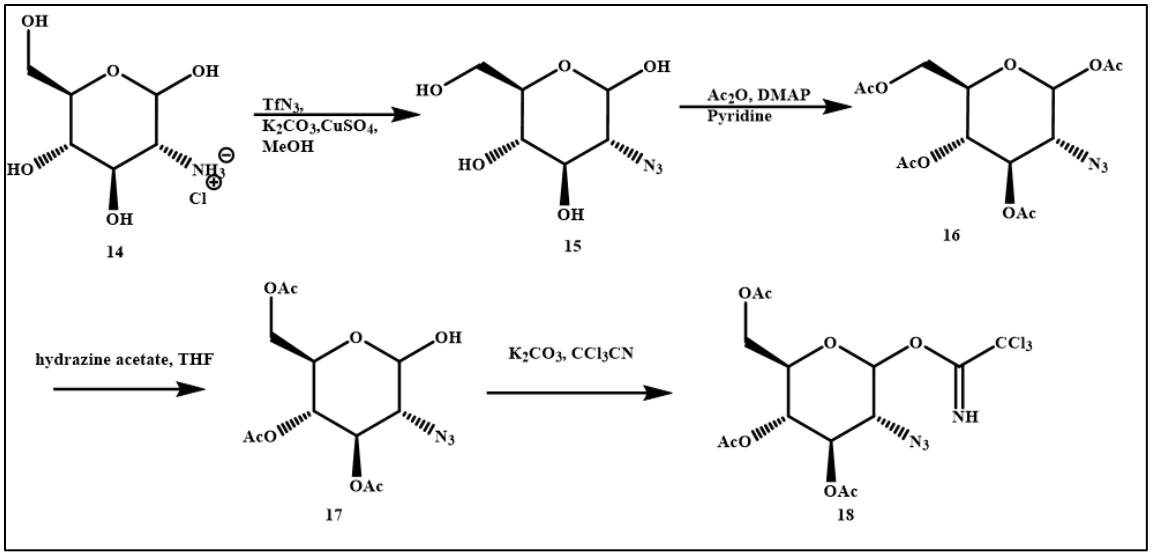


Figure 9. Synthesis of glucosamine donor

Figure 9 shows the synthesis of the glucosamine donor, compound **18**, from glucosamine hydrochloride. It was a similar procedure for the monosaccharide glycosyl donor except the final step was to covert the anomeric hydroxide to a trichloroacetimidate.[31]

The uronic acid donors were made from diacetone-D-glucose as shown in Figure 10. The 3-hydroxide was permanently protected by benzyl group to yield compound **20**, which is the precursor for glucuronic acid. Then the isopropylidene was deprotected by acetic acid and the only exposed primary hydroxide was protected by pivaloyl group.[32] The only free hydroxide group was then protected by triflyl group and went through a stereo-reversion by an SN_2 mechanism to afford compound **24**, which was then deprotected to yield compound **25**, the precursor for the iduronic acid donor. The uronic acid donors were then converted to the pyranose (compound **26** and **27**) form by sulfuric acid at 80 ºC. Final uronic acid donors (compound **35** and **37**) were obtained within five steps: acetylation, thiolation, acetyl ester hydrolysis, benzylidene protection and acetylation.[31] A linker, compound **38**, was then attached to the uronic acid donors via a thiol glycosylation and then through deprotection of benzylidene and oxidation of hydroxyl group, the final uronic acid donors, compound **41** and **44**, were

achieved as shown in

Figure 11. The glucosamine donor and uronic acid donors were conjugated together via glycosylation with triflic acid as the promoter. A new aglycone for the disaccharide substrate had been synthesized as shown in

Figure 11. All procedures were identical to the aglycone synthesis of monosaccharide substrate with a final step modification. The hydroxyl group was converted to a N-hydroxysuccinimide ester in compound **51** to form an amide between the disaccharide linker and the aglycone. As shown in Figure 12, compound **45** and **46** were deprotected to expose all hydroxyl group except for the 3-position on the uronic acid, which was protected by benzyl group, by base catalyzed hydrolysis. Then, the azido group was converted to an amine by Staudinger reaction with trimethylphosphine to yield compound **54** and **56**. These two compounds then went through selective sulfation and hydrogenation to afford compound **56** and **57**, which were subjected to a reaction with compound **51** to achieve the final MPS III A disaccharide substrates.[33]

The general structure of heparan sulfate indicates that the repeating disaccharide unit has a glucosamine and an uronic acid. The uronic acid can be either iduronic acid or glucuronic acid. The new disaccharide substrates have two variations: N-sulfate-glucosamine-iduronic acid-aglycone (GlcN-IdoA) and N-sulfate-glucosamine-glucuronic acid-glycone (GlcN-GlcA).

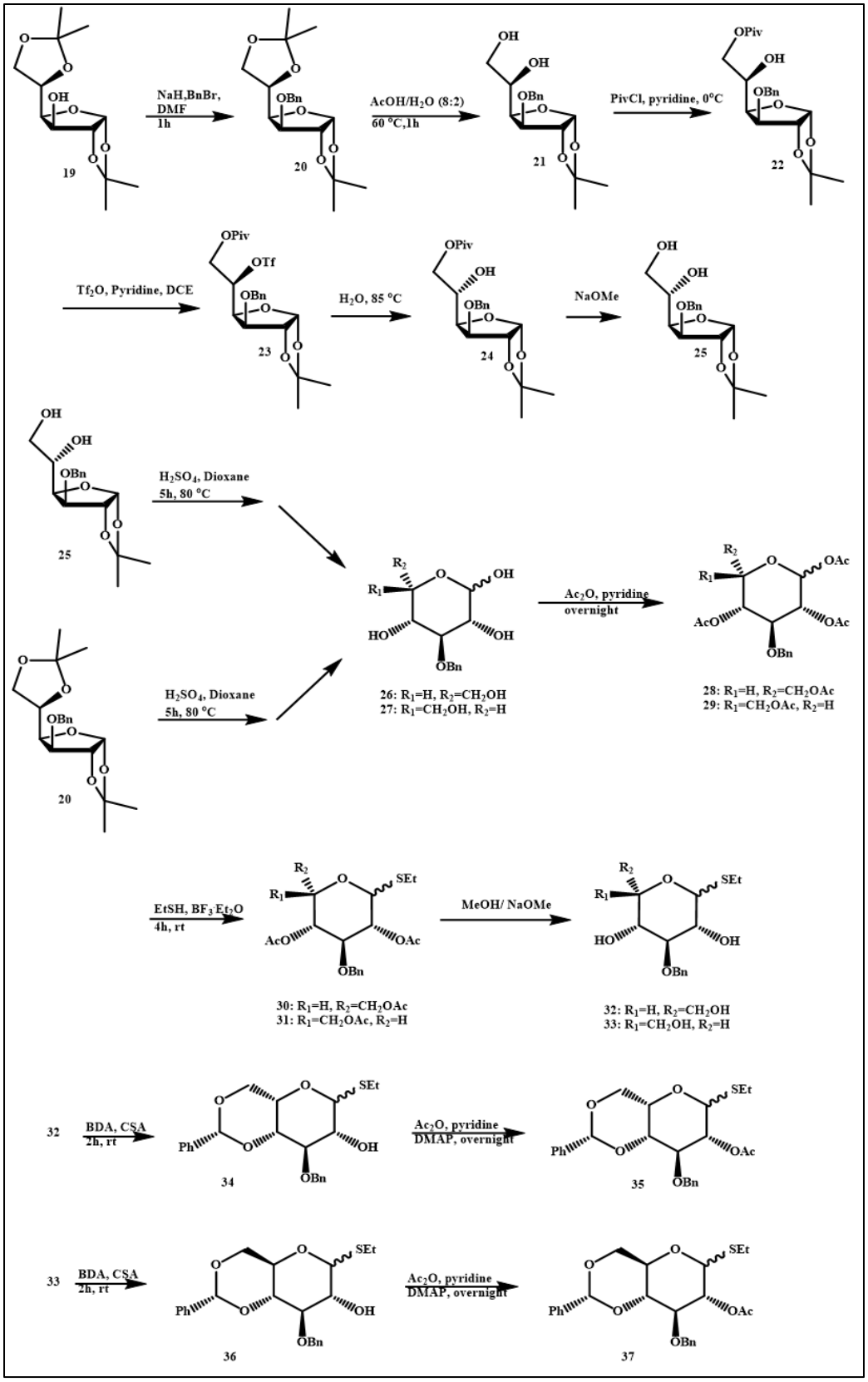


Figure 10. Synthesis of uronic acid donor

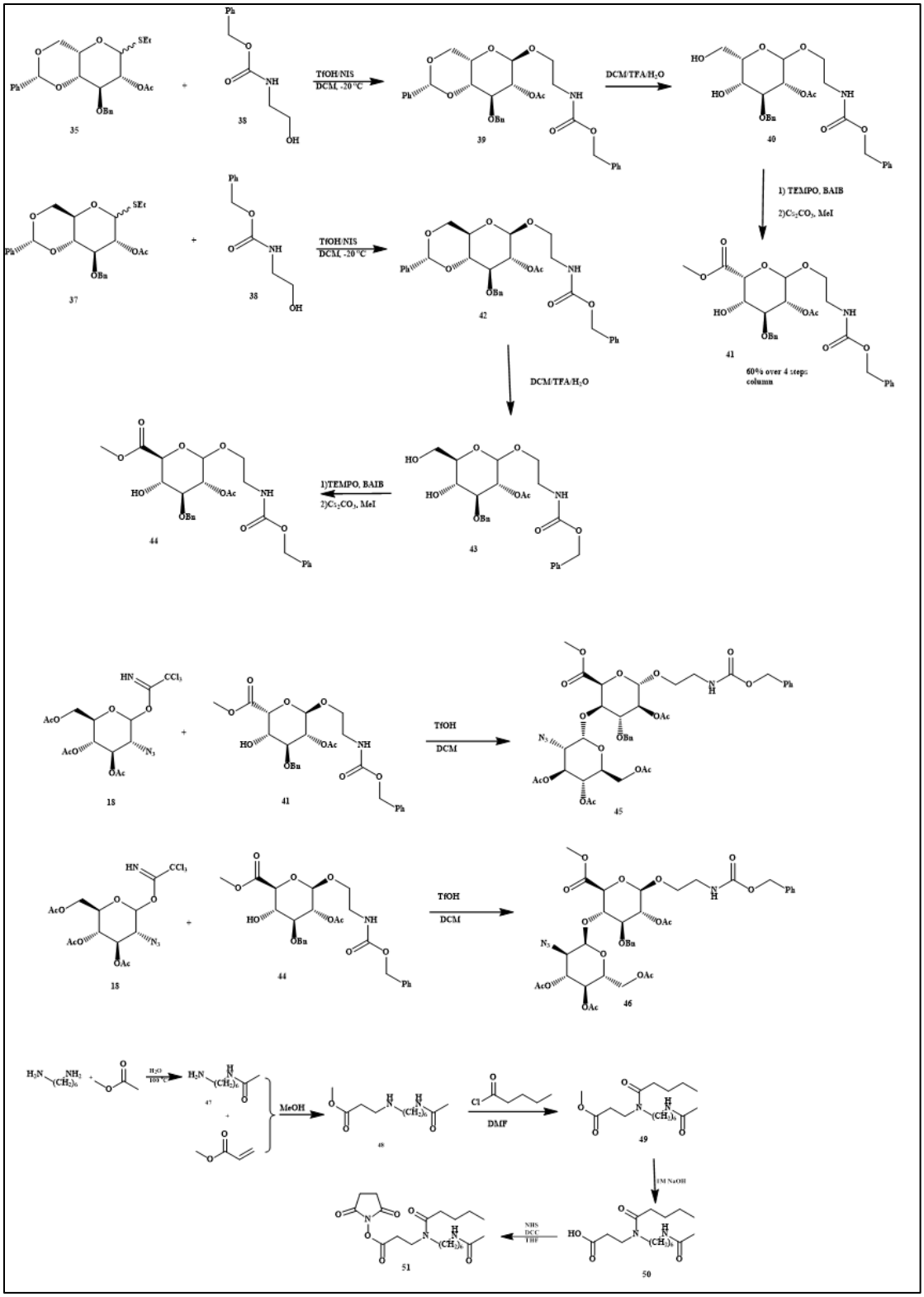


Figure 11. Synthesis of disaccharide unit and aglycone for sulfamidase substrate

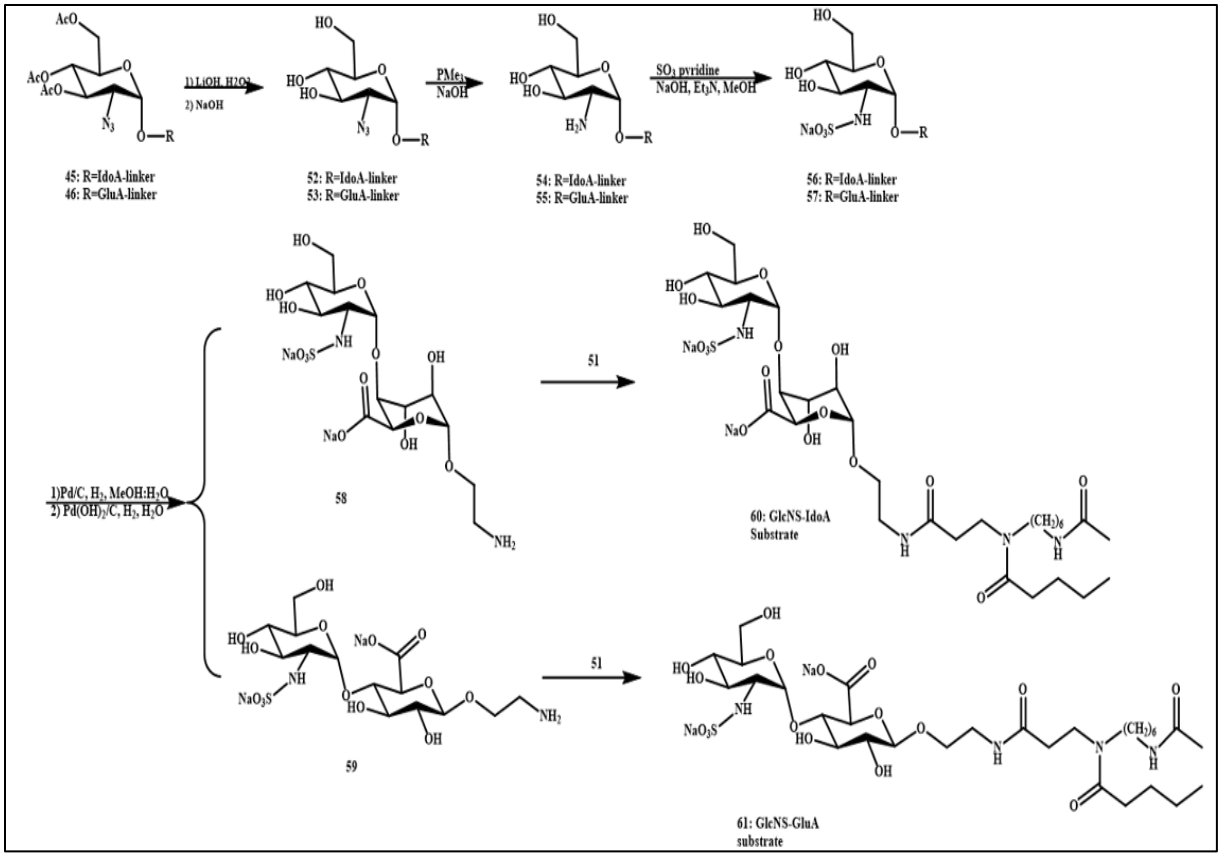


Figure 12. Synthesis of the final disaccharide sulfamidase substrate

3.2.2. Enzymatic assay with second-generation sulfamidase substrate

With previous reports about sulfamidase assay[5,16], the initial assay buffer condition was established as follow: 50 mM sodium acetate, 7.5 mM barium (II) acetate, 5 mM cerium (III) acetate and pH 5.5. The assay used a 3 mm DBS punch (equivalent to 3.2 µL of blood) in a 96 deep well plate. To each DBS punch, 30µL of buffer containing 1 mM MPS III A substrate was added and put in the 37°C orbital shaker for 16 hours at 250 rpm. The assay was then quenched by adding 120 µL of acetonitrile to each well, followed by 5 minutes of centrifugation at 3000 rpm to pellet the precipitate. 120 µL of the supernatant was then transferred to a shallow well plate and 120µL water was added to the plate. Then the plate was sealed with aluminum foil and subjected to LC/MS/MS directly. Mass spectrometry was performed on a Waters Xevo

instrument using positive ion mode selected reaction monitoring (SRM) with tuned parameters that optimize the signal from the enzymatic product.

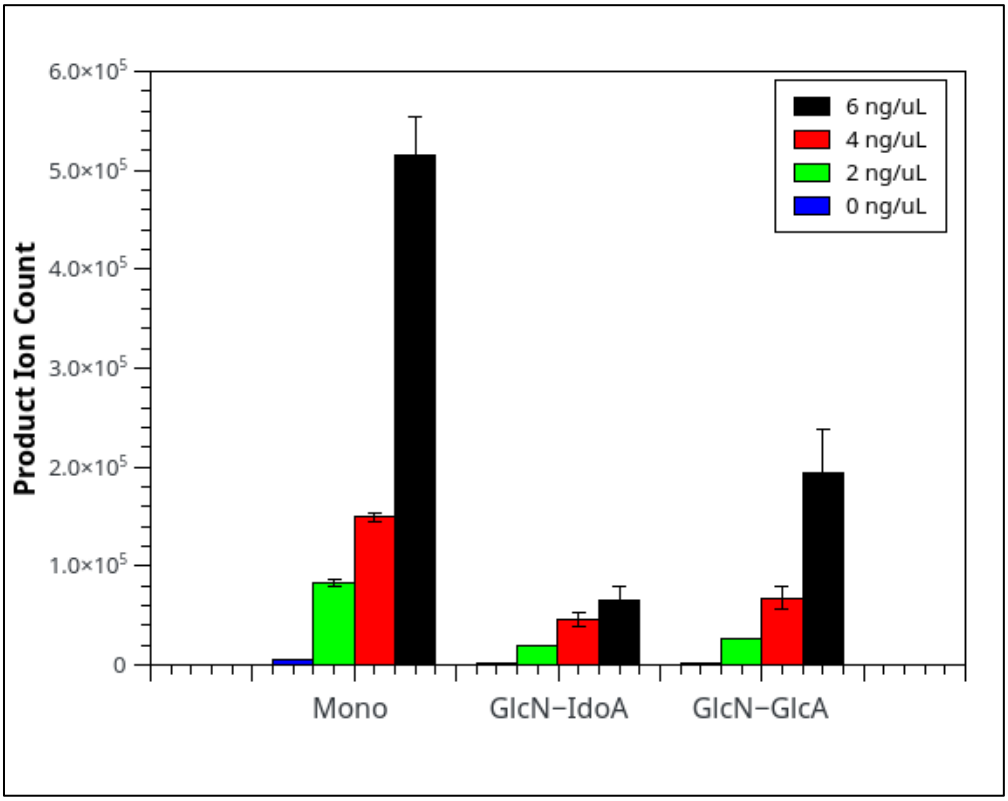


Figure 13. Comparasion between monosaccharide substrate to disaccharide substrates under various concentration of rhSGSH. (Mono: monosaccharide substrate; GlcN-IdoA: disaccharide substrate with iduronic acid; GlcN-GlcA: disaccharide substrate with glucuronic acid).

To address the question of whether the newly synthesized disaccharide substrates are viable substrates for sulfamidase like the first-generation substrate, an experiment was conducted with rhSGSH. 1 mM substrates (all three substrates made for sulfamidase so far) was exposed to different concentrations of the recombinant sulfamidase (2 ng/μL, 4 ng/μL and 6 ng/μL). As shown in Figure 13, the disaccharide substrates could indeed be recognized by the rhSGSH as there was a positive correlation between the concentration of recombinant enzyme and product formed (represented by the product ion count); however, it also shown that the newly synthesized

disaccharide substrates did not produce as much product signal as the monosaccharide substrate did. In fact, there was at least a two-fold decrease in product signal from the disaccharide substrate to the one from the monosaccharide substrate. Although this data was obtained by rhSGSH, it could still suggest that these disaccharide substrates were not as efficient as previously reported in the literature for sulfamidase activity.

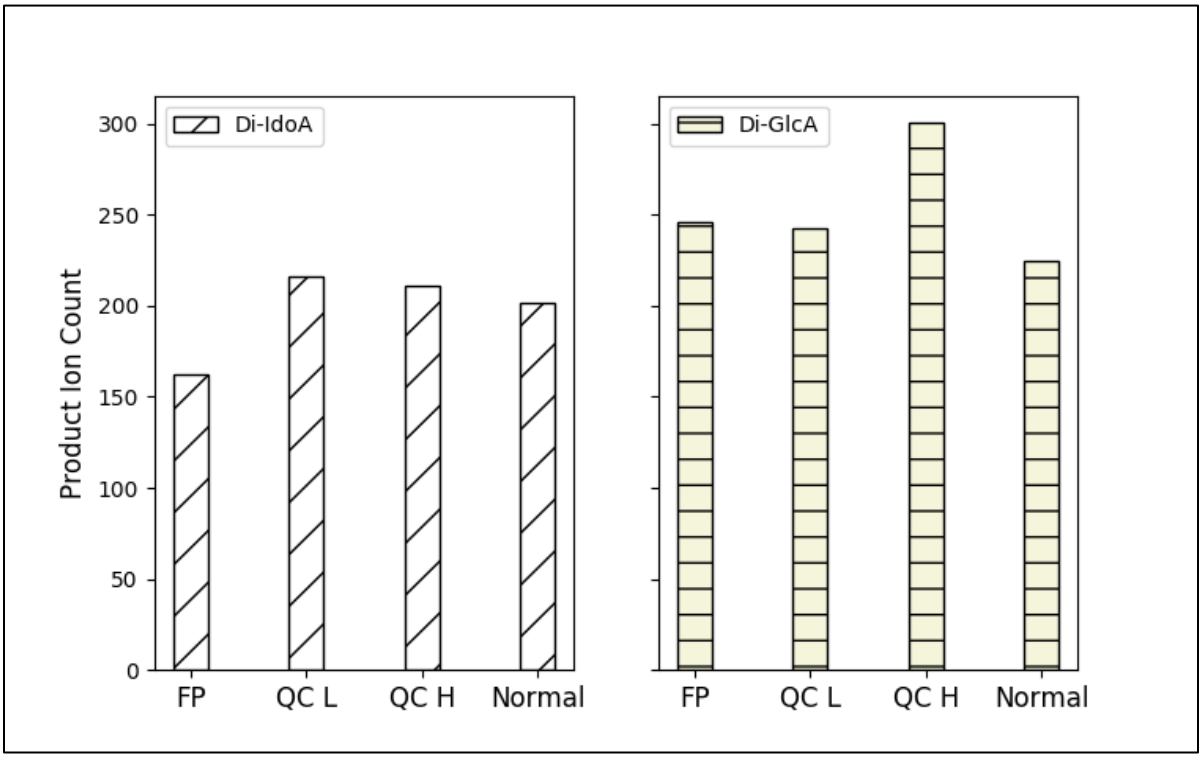


Figure 14.DBS assay with disaccharide substrates. FP: filter paper blank; QC L: quality control low; QC H: quality control high; Normal: random normal DBS.

In the hope of DBS assay may have a different result, quality control DBS and a normal DBS was subjected to the enzymatic assay in condition mentioned above. As indicated in Figure 14, there was no corresponding signal in different DBS samples as one would expect for a viable assay. One can argue that for glucuronic-disaccharide substrate, there was an increase in product formation from QC L to QC H; however, the 50 counts increase did not justify that this is a real result from sulfamidase action instead of a normal MS response fluctuation. Going back to

Hopwood's paper[30], they stated that the disaccharide substrates were significantly better than monosaccharide substrate in the sense of catalytic efficiency. However, the same rule may not apply to a DBS assay here, because of the excessive amount of substrate that was used in the assay. When the concentration of the substrate was always saturating, all that matters is the V_{max} of the enzymatic reaction. The goal should be finding a substrate that has maximum V_{max} when working with sulfamidase.

3.3. The third generation sulfamidase substrate with a fused aromatic aglycone

3.3.1. Synthesis of third generation sulfamidase substrate

In 1996, Karpova and coworkers published a sulfamidase substrate for fluorometric assay in leukocytes and fibroblast.[18] This substrate has a N-sulfated glucosamine alpha 1-4 linked to a 4-methylumbelliferone (4MU). The 4MU substrate for sulfamidase has similar V_{max} as the one for a tetra-saccharide substrate[34], but the K_m of 4-MU substrate (4 mM) is significantly higher than that of tetra-saccharide substrates (10 – 30 μM).[30] This suggested that there is no absolute requirement for an adjacent sugar residue present in the sulfamidase substrate for a high V_{max}; the structural similarity to the natural substrate for sulfamidase is only required for a small K_m. Since the goal is to find a substrate with a high V_{max}, a new design for the sulfamidase substrate can start with altering the aglycone structure. Although the K_m will be higher than the one for the polysaccharide substrate, a higher concentration of substrate is affordable since the synthesis of a monosaccharide substrate is vastly easier than the one for polysaccharide substrates. The 4-MU substrate shows a similar V_{max} as a tetra-saccharide substrate. It is a good starting point for the discovery of the new sulfamidase substrate.

4-MU substrate itself proved not to be a good substrate in DBS assay when both 1 mM and

10 mM of this substrate was used in the incubation (Table 1). The product signal produced from the assay of both concentrations of substrate were similar, which is contradictory to the idea that the K_m for 4-MU substrate is around 4 mM. Further investigation of this substrate with rhSGSH suggests that the 4-MU substrate was not a better substrate than the three substrates mentioned above in a DBS assay. Also, the claimed 3-4 folds in activity increase for sulfamidase with pefabloc present in the buffer was not observed.

Table 1. The DBS assay with 4MU substrate in 2 different concentrations of substrate. (original buffer is the MPS III A DBS buffer mentioned above; the pefabloc buffer contains 0.1 mg/mL pefabloc, a protease inhibitor, in the original buffer)

		GlcNS-4MU product count	
		1mM [S]	10mM [S]
original buffer	FP	12.09	39.80
	DBS	252.11	165.97
	SGSH2	7378.13	16351.14
pefabloc buffer	FP	13.50	14.77
	DBS	346.13	194.69
	SGSH2	6947.02	19222.23

To start off the aglycone modification, a 2-naphthol was conjugated to compound **5** with boron trifluoride diethyl etherate as the promoter to yield compound **62**. Then, acetyl protections were hydrolyzed by sodium hydroxide to afford compound **63**, which was then undergo a Straudinger reaction with trimetylphosphine to obtain compound **64**. Finally, the third generation sulfamidase substrate, compound **65** (GlcNS-2-Nap), was obtained by sulfation with sulfur trioxide pyridine complex.

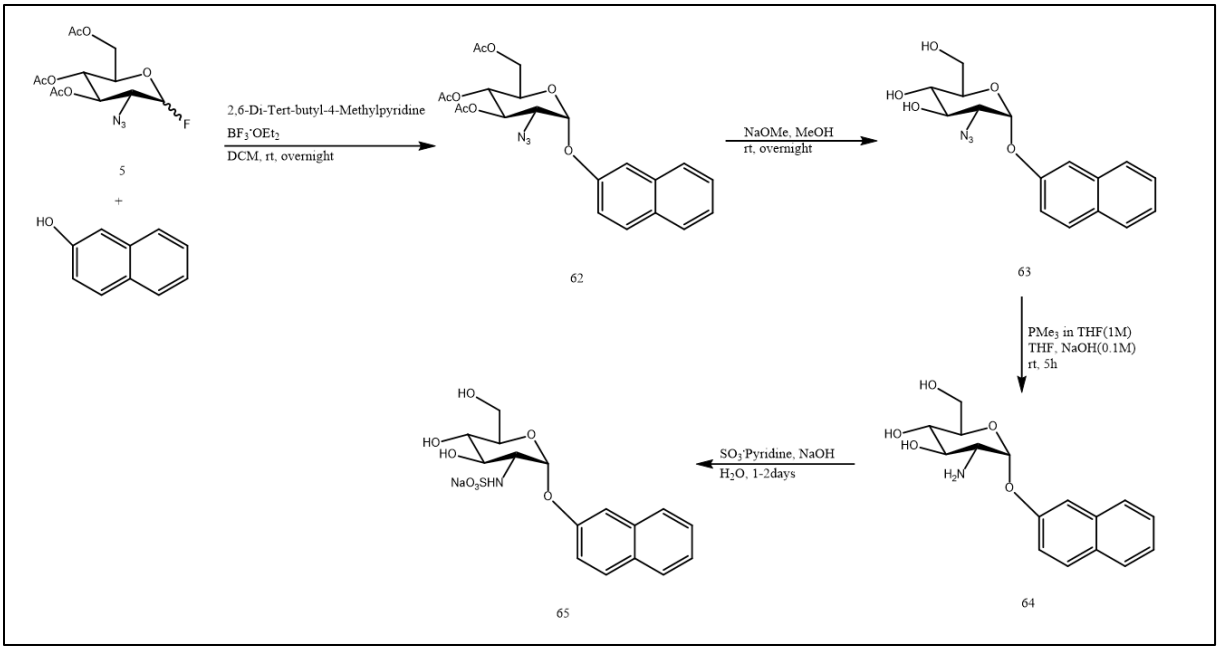


Figure 15. Synthesis of the final third generation sulfamidase substrate

3.3.2. Assays condition optimization with GlcNS-2-Nap

Surprisingly, the first attempt of aglycone modification yields a result that was never seen before.

The activity of the enzyme was calculated by the following formula:

$$Activity(\frac{\mu mol}{h \cdot L}) = \frac{\left(\frac{P}{IS}\right) \times [IS] \times V_{IS}}{t_{inc} \times V_{blood}}$$

IS stands for internal standard and it was made by using a d7 deuterium labeled 2-Naphthol-1,3,4,5,6,7,8-d_7 instead of the original 2-naphthol. In this way, the IS has a different mass than that of the substrate while it retains all the chemical properties of the latter. In the field of MS, an human error if it is placed with the quantifier at the beginning of the experiment. The activity (in unit of µmole/h/L) can be achieved by using the ratio between the product signal (P) to IS signal

(IS) multiplying the amount of IS put into the assay ([IS]× V_{IS}); IS must be used to achieve a quantitative result. The IS will account for all the sample lost and then, divide by time (t_{inc}) and volume of blood (V_{blood})in one 3 mm DBS punch (3.2 µL).

As usual, a rhSGSH assay was conducted first to confirm the viability of this substrate to sulfamidase. As shown in Figure 16, with the same concentration of rhSGSH (2 ng/µL), GlcNS-2-Nap showed over 1 million product ion count versus the mono- and disaccharide substrates showed less than 100,000. When considered that GlcNS-2-Nap has a less MS sensitive aglycone structure than the others, the 10-fold increase in product signal was even more significant. This finding also confirmed that the aglycone structure plays a crucial role in sulfamidase V_{max}.

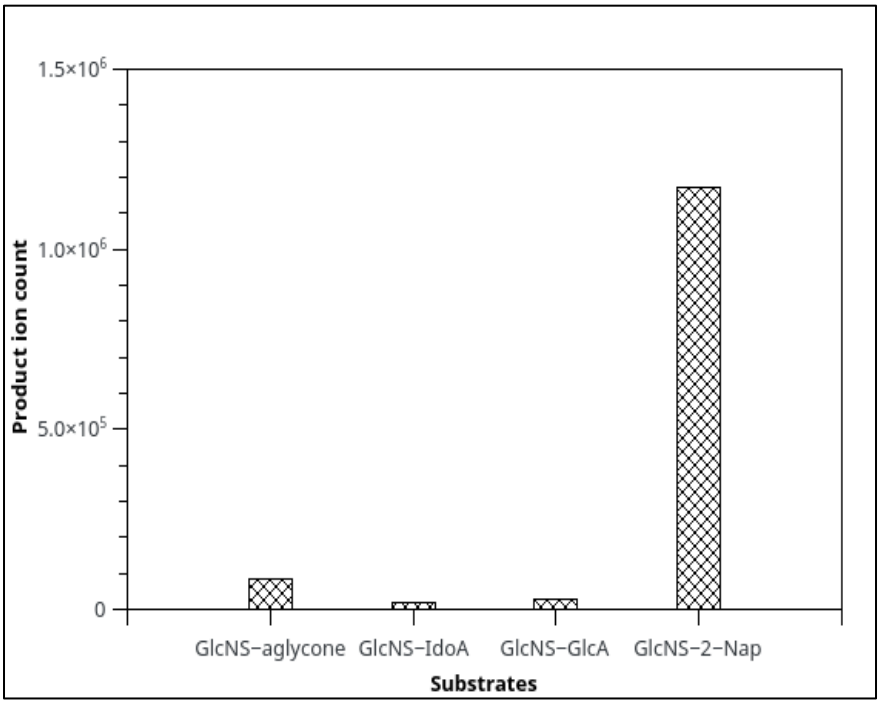
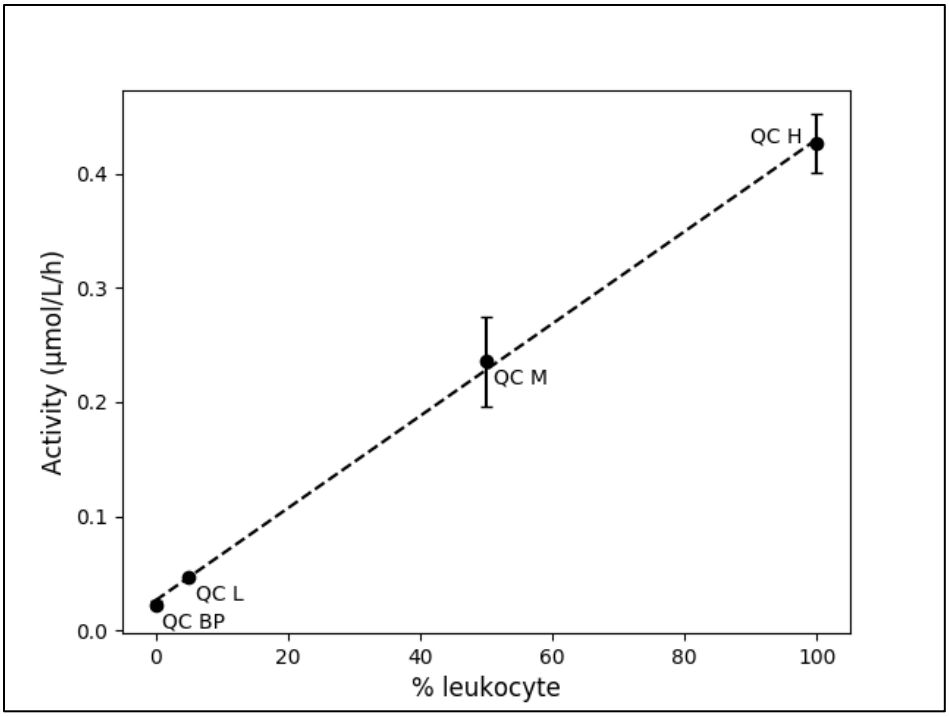


Figure 16. Product signal from an assay with 2 ng/µL of rhSGSH for the three generations of substrates. (GlcNS-aglycone: monosaccharide substrate; GlcNs-IdoA: disaccharide substrate with iduronic acid; GlcNs-GlcA: disaccharide substrate with glucuronic acid; GlcNS-2-Nap: the third generation sulfamidase substrate)

The Data obtained from the rhSGSH assay has shown promising result for the new substrate. Next, an experiment was conducted with quality control DBS to confirm that the new substrate could be used to differentiate different DBS samples (Figure 17). A linear relationship was observed for the sulfamidase activity versus the fraction of whole blood in the QC standards. One can also observe the differentiation in sulfamidase activity between QC BP and QC L, which means the assay was able to tell when there was only 5% residual sulfamidase activity in DBS. The usual cutoff value for a screening process is around 10-15%, so this new assay was able to go below 5% shows its usefulness in the screening process. This is the first time that any substrate has shown a correlation between the activity and the percent of leukocyte for MPS III A DBS assay.

CDC QC DBS has some artificial characteristics in it, it does not exactly represent the behavior of a true DBS. When a DBS from a healthy adult was used in the assay, the sulfamidase activity was 0.176 μmol/L/h compared to 0.008 μmol/L/h when a filter paper (FP) punch (no blood) was submitted to the same assay protocol (Figure 18). This 22-fold difference is lower than the blood-to-no blood ratios seen with other MS/MS assays of lysosomal enzymes[11] in DBS (usually above 90) but still adequate for screening as it is larger than values typically seen with fluorimetric assays of the same enzymes (usually below 10).[10] It was also observed in this study that wild-type mouse has a much higher sulfamidase activity (9.77 μmol/L/h); while the MPS III A affected mouse still shown low activity (0.013 μmol/L/h) which was close to the FP activity level. The reason for the high sulfamidase activity in wild-type mouse is unknown.



Sample	Activity (µmol/L/h)	B/noB	SD	CV%
QC BP	**0.022**	**2.6**	**0.0033**	**14.9**
QC L	**0.046**	**5.5**	**0.0015**	**3.3**
QC M	**0.236**	**27.7**	**0.0389**	**16.5**
QC H	**0.427**	**50.3**	**0.0252**	**5.9**

Figure 17. Sulfamidase assay with CDC quality control DBS sample using the new substrate (GlcNS-2-Nap). QC BP: quality control base pool with 0% normal leukocyte; QC L: quality control low with 5% normal leukocyte; QC M: quality control medium with 50% normal leukocyte; QC H: quality control high with 100% normal leukocyte; B/noB: Blood to no blood ratio. Table shows the actual numeric data for figure 17.

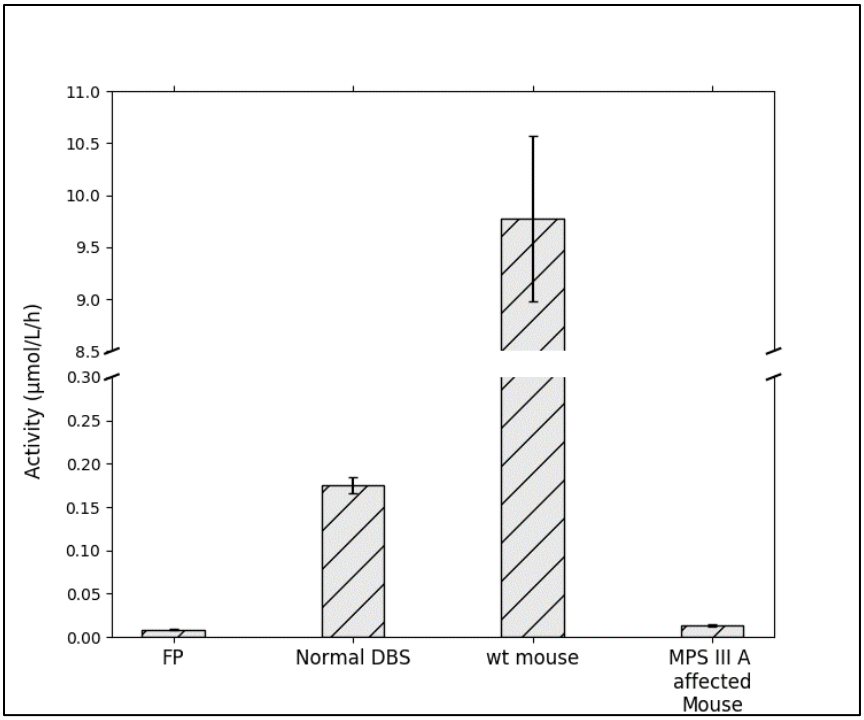
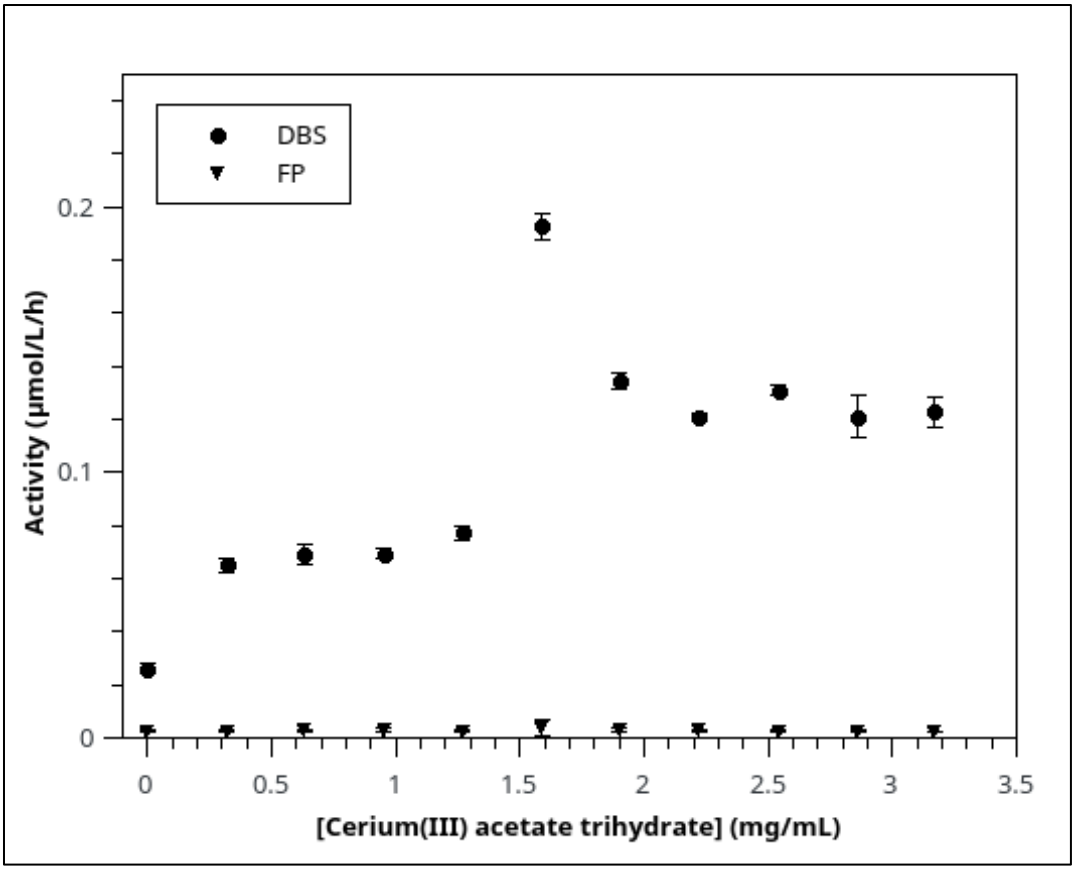


Figure 18. PS III A assay with normal DBS, wild type mouse DBS, and MPS III A affected mouse DBS. (FP activity: 0.008 µmol/L/h; Normal DBS activity: 0.176µmol/L/h; wt mouse DBS activity: 9.77 µmol/L/h; MPS III A affected mouse DBS activity: 0.013 µmol/L/h)

The next goal was to establish the optimal condition for MPS III A assay in DBS. The original buffer contains both cerium and barium additives which were used to precipitate sulfate and phosphate, respectively. However, later study has shown that cerium itself was sufficient in blocking the prohibition effect from sulfate and phosphate together.[11] To optimize the concentration of cerium in the DBS assay, a series of buffer was made with different concentrations of cerium (III) acetate hydrate.



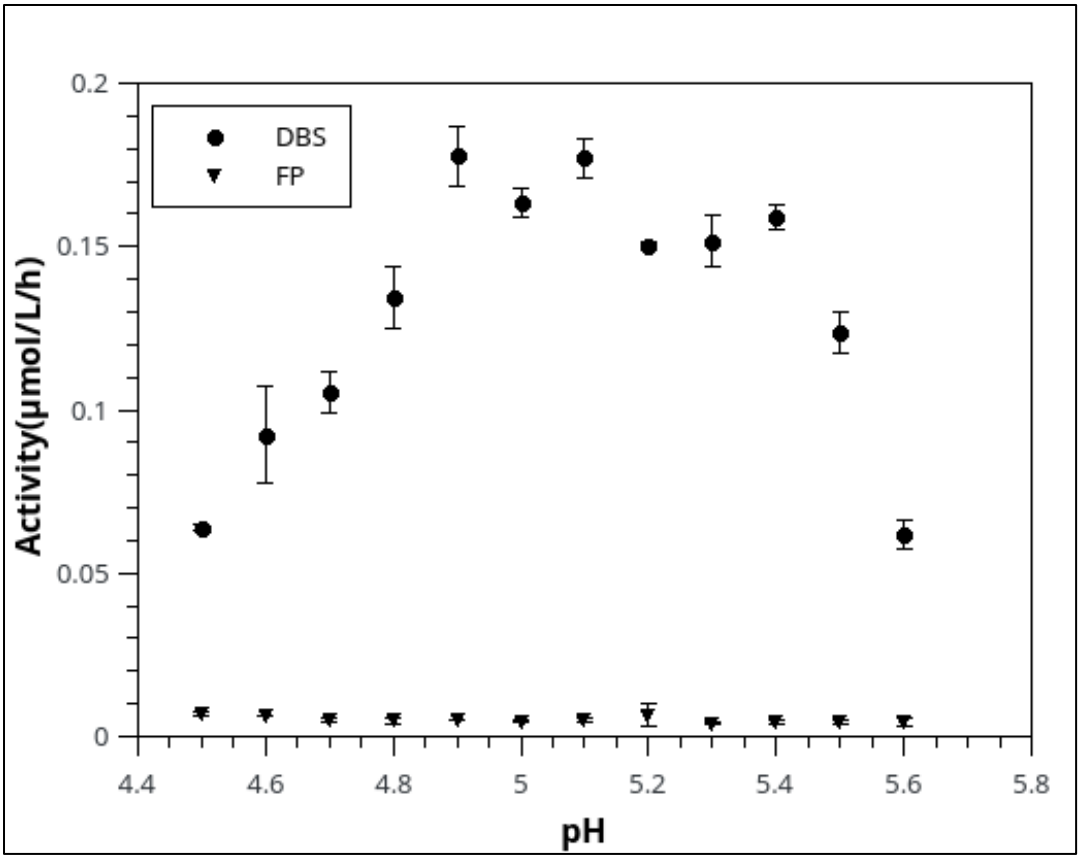
	activity(µmol/L/h)		SD		%CV	
[Ce](mg/mL)	FP	DBS	FP	DBS	FP	DBS
0.00	0.0025	0.0260	0.0003	0.0019	11.8	7.4
0.32	0.0026	0.0652	0.0001	0.0027	4.0	4.2
0.63	0.0028	0.0692	0.0003	0.0038	11.9	5.5
0.95	0.0029	0.0693	0.0007	0.0020	25.9	2.8
1.27	0.0025	0.0774	0.0005	0.0027	18.8	3.4
1.59	0.0039	0.1928	0.0028	0.0049	70.7	2.6
1.90	0.0031	0.1342	0.0007	0.0031	24.0	2.3
2.22	0.0028	0.1208	0.0005	0.0012	18.3	1.0
2.54	0.0026	0.1308	0.0002	0.0019	6.4	1.5
2.86	0.0025	0.1210	0.0002	0.0081	8.9	6.7
3.17	0.0024	0.1228	0.0001	0.0057	6.1	4.7

Figure 19. Cerium (III) acetate concentration optimization in MPS III A DBS assay. Table shows the actual data. Large CV is to be expected for FP samples.

As summarized in figure 19, the optimized cerium (III) concentration appeared to be 1.59 mg/mL in assay buffer. The exact molar concentration is unknown due to the unknown molecular weight of the cerium acetate hydrate.

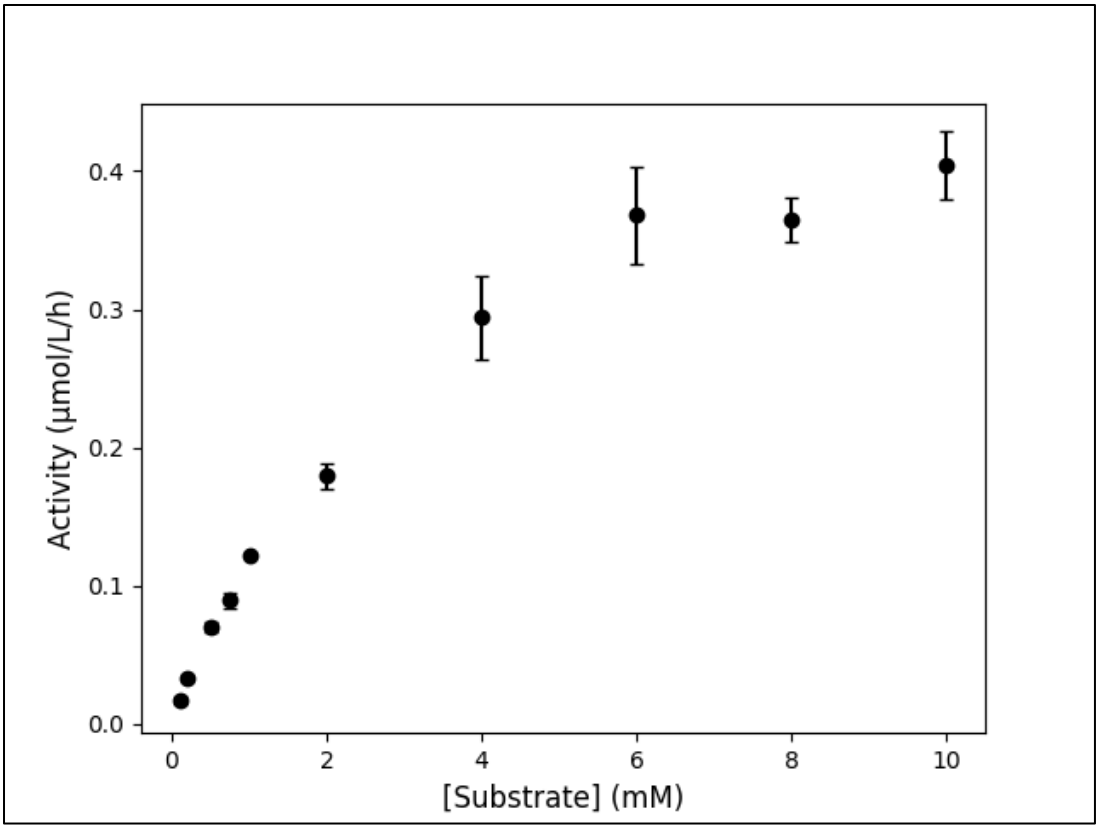
Next, the pH of the assay buffer was optimized in the range from 4.5 to 5.6 as that is the range for sulfamidase to function in the literature. The buffers were prepared with 1.59 mg/mL cerium (III) acetate trihydrate and 50 mM sodium acetate. Then, the pH was adjusted with a pH meter, calibrated with pH 4.0 and pH 7.0 standard solution, using acetic acid and sodium hydroxide. As shown in Figure 20, sulfamidase in DBS appears to have an optimal pH around pH 5.0 and the activity decreases dramatically outside the optimal pH range. This finding is consistent with the value reported in the literature.[30,35]

As mentioned above, when there is no adjacent sugar residue present next to the glucosamine in the sulfamidase substrate, the K_m increase dramatically. To investigate how much substrate should be used in the assay, an assay with various substrate concentrations was conducted. As summarized in figure 21, the activity of sulfamidase in one DBS punch starts to plateau after 6 mM substrate concentration. From a Lineweaver-Burk plot (figure 22), a function between 1/(enzyme activity(V)) and 1/[substrate] was obtained as $1/V = 5.742(1/[S]) + 2.3413$. From this equation, K_m could be calculated as 2.45 mM and V_{max} was 0.42 μmole/h/L. The K_m for the new substrate is lower than the 4-MU substrate, but it is still a higher concentration that one would want to pass through the MS because this is no easy way to do the post assay workup to get rid of the substrate. From Figure 20, the conclusion was made that at 1 mM substrate concentration, the sulfamidase activity was strong enough for a screening process, so from now on all future assay was ran with 1 mM substrate.



	Activity (µmol/L/h)		SD		%CV	
pH	FP	DBS	FP	DBS	FP	DBS
4.5	0.0068	0.0640	0.0005	0.0012	7.0	1.8
4.6	0.0064	0.0923	0.0001	0.0147	1.5	16.0
4.7	0.0053	0.1054	0.0006	0.0064	11.8	6.1
4.8	0.0048	0.1342	0.0010	0.0095	21.3	7.0
4.9	0.0050	0.1777	0.0001	0.0093	3.0	5.2
5.0	0.0045	0.1633	0.0003	0.0044	5.5	2.7
5.1	0.0050	0.1770	0.0008	0.0059	15.6	3.3
5.2	0.0065	0.1503	0.0033	0.0012	51.0	0.8
5.3	0.0041	0.1516	0.0003	0.0080	6.1	5.2
5.4	0.0046	0.1590	0.0006	0.0036	13.1	2.2
5.5	0.0045	0.1236	0.0006	0.0065	13.3	5.3
5.6	0.0043	0.0620	0.0012	0.0044	28.3	7.1

Figure 20. pH optimization of MPS III A DBS assay. Table shows the actual data. Large CV is to be expected for FP samples. There is a plateau starting from pH 4.9 to pH 5.1 in blood to no blood ratio. pH 5.0 is chosen as the assay running pH.



	Activity (µmol/L/h)		SD		%CV	
[substrate] (mM)	FP	DBS	FP	DBS	FP	DBS
0.1	0.0041	0.0208	0.00077	0.00278	18.8	13.4
0.2	0.0073	0.0399	0.00063	0.00146	8.7	3.7
0.5	0.0166	0.0865	0.00130	0.00362	7.8	4.2
0.75	0.0245	0.1136	0.00177	0.00503	7.2	4.4
1	0.0348	0.1568	0.00349	0.00043	10.0	0.3
2	0.0667	0.2460	0.00329	0.00883	4.9	3.6
4	0.1340	0.4281	0.00519	0.03040	3.9	7.1
6	0.1900	0.5580	0.01081	0.03507	5.7	6.3
8	0.2540	0.6192	0.00254	0.01586	1.0	2.6
10	0.3438	0.7477	0.02453	0.02453	7.1	3.3

Figure 21. Substrate concentration curve in MPS III A DBS assay with GlcNS-2-Nap. Table shows the actual data. There is a plateau starting from 6 mM substrate.

Further study was conducted regarding the incubation time of MPS III A DBS assay. As shown in Figure 23, there was a linear relationship between the incubation time (hours) and the product formed (pmole). Also, as the time increased, there was no significant non-enzymatic

product formation, meaning the FP blank activity remained low. To accommodate the need for a newborn screening lab, the incubation time was set at 16 hours as it can be left overnight for next day processing.

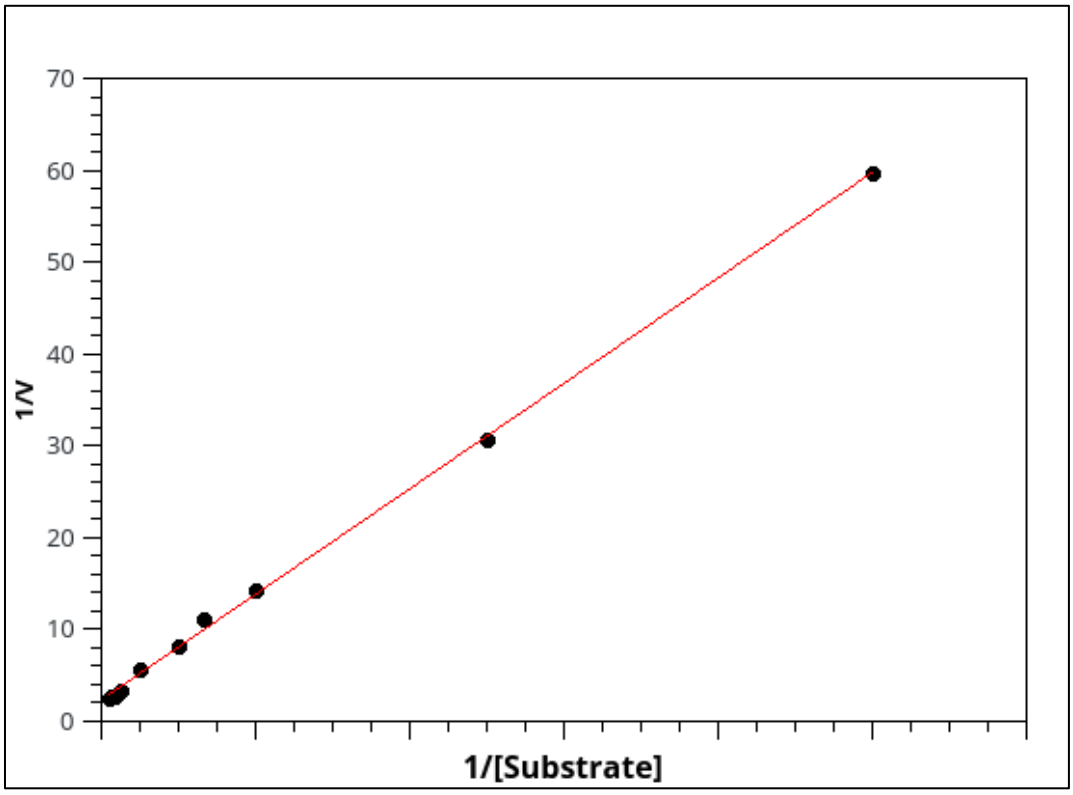
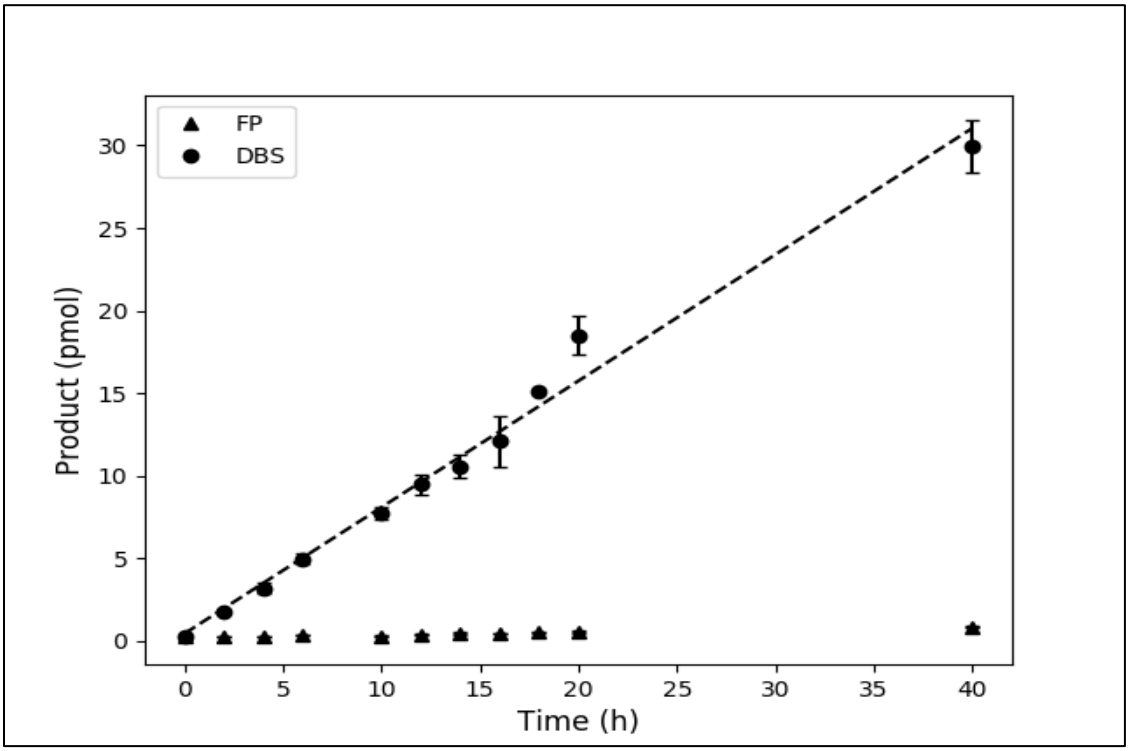


Figure 22. Lineweaver-Burk plot of sulfamidase in DBS assay. Y-axis: 1/V, V is sulfamidase activity; x-axis: 1/[substrate]; 1/V = 5.742(1/[S]) + 2.3413. Hence, K_m could be calculated as 2.45 mM and V_{max} was 0.42 µmole/h/L.

3.3.3. Final DBS assays with GlcNS-2-Nap

Now, the final condition for MPS III A DBS assay was set as follow: 1.59 mg/mL cerium (III) acetate, 50 mM sodium acetate, pH 5.0, 1 mM substrate concentration, 1 µM internal standard. The assay started with 30 µL assay cocktail mentioned above with one 3 mm DBS punch in a shallow 96 well plate. The plate was centrifuged in a swinging bucket rotor for ~1

min at ~2095 rcf to ensure that the DBS was fully immerged and all the liquid was at the bottom of the well.



	Product formed(pmol)		SD		CV%	
time(h)	FP	DBS	FP	DBS	FP	DBS
0	0.2247	0.2162	0.09652	0.0559	43.0	25.8
2	0.2387	1.7859	0.00120	0.0960	0.5	5.4
4	0.2568	3.1617	0.04038	0.3191	15.7	10.1
6	0.3454	4.9518	0.03923	0.3352	11.4	6.8
10	0.3039	7.7205	0.01096	0.3450	3.6	4.5
12	0.3818	9.4625	0.03022	0.5841	7.9	6.2
14	0.4782	10.5713	0.08467	0.6606	17.7	6.2
16	0.4429	12.0760	0.01687	1.5785	3.8	13.1
18	0.5203	15.0957	0.04420	0.1118	8.5	0.7
20	0.5444	18.4907	0.09873	1.1733	18.1	6.3
24	0.5912	16.3337	0.06463	2.5554	10.9	15.6
40	0.8571	29.9231	0.07929	1.5768	9.3	5.3

Figure 23. The incubation time for MPS III A DBS assay. Table shows the actual data. Large CV is to be expected for FP samples. In a period of 40 hours, sulfamidase activity does not slow down.

The plate was seal with a silicone sealing matt, and then placed on an orbital mixing platform (~250 rpm) at 37 °C for 16 hr. Enzymatic reactions were quenched by addition of 100

mL of acetonitrile, and the plate was centrifuged for 5 min at room temperature at ~ 2095 rcf. A portion of the supernatant (90 mL) was transferred to an autosampler plate, and it was dried by a stream of nitrogen gas (or jet of oil-free air). The dried sample was then reconstituted with 90 µL of 90/10 H_2O/ACN. The plate was then wrapped with aluminum foil to minimize solvent evaporation and placed in the cooled (8 °C) autosampler chamber of the LC-MS/MS instrument.

The column used in this study was ACQUITY CSH C18 UPLC, 1.7 µm, 2.1mm x 50 mm with a guard column (Waters Cat. 186005296 and 186005303). The column temperature was set at 40 °C. Solvent A was water/acetonitrile (90/10) with 0.1 % formic acid (all LC solvents are Optima grade from Fisher Scientific). Solvent B was acetonitrile/isopropanol (50/50) with 0.1% formic acid. The elution gradient in the LC was programmed as: 0-0.3 min (3% solvent B), 0.3-0.94 min (3% to 60% solvent B, linear gradient), 0.95-1.64 min (100% solvent B, step change at 0.95 min), 1.65-2.0 min (3% solvent B, step change at 1.65 min). The flow rate was 0.5 mL/min. For the autosampler, the weak needle wash was water/acetonitrile (90/10) with 0.1 % formic acid, and the strong needle wash was ACN with 0.1% formic acid.

MS/MS was carried out on a Waters Xevo-TQ instrument equipped with a Waters Acquity UPLC system. MS/MS instrument settings are given in Table 2. Sulfamidase activity (µmole/h/L) was obtained by multiplying the ratio of ion counts of sulfamidase product to that of the internal standard by the µmole of internal standard added to the assay, then dividing by the incubation time (h) and volume of blood in one 3 mm DBS punch (3.2uL).

A typical chromatogram of MPS III A DBS assay was shown in Figure 24, the top two panels represent the IS channels for patient and normal newborn, respectively. The bottom two panels represent the product channels for newborn and patient, respectively. The IS is eluting 0.02 min before the actual product even though it should have the same chemical properties and

chromatographical behavior. This is caused by the 7 deuteriums in the IS. The heavier IS is more susceptible to elution by the solvent as established by the MS community. Nonetheless, the elution times between the product and IS are close enough to think that the IS can represent the product as if they are equal in the whole assay.

Table 2. Mass spectrometer parameter for MPS III A DBS assay with GlcNS-2-Nap

Parameter (units)	**Value**
Polarity	ES+
Capillary Voltage (V)	3500
Extractor (V)	3.0
Source temperature (°C)	150
Desolvation temperature (°C)	400
Cone gas flow (L/hr)	30
Desolvation gas flow (L/hr)	1000
LM 1 resolution	3.0
HM 1 resolution	15.0
Ion energy 1	0.0
Collision cell entrance potential (V)	0.5
Collision cell exit potential (V)	0.5
LM 2 resolution	2.8
HM 2 resolution	15.0
Ion energy 2	0.6
Multiplier (V)	493.04
Collision gas	Nitrogen

The substrate was eluting around 1.2 min as indicated by the dash line box in Figure 24. The reason that there was no substrate peak was that all eluent was directed to the waste after 0.9 min to minimize the impact of high concentration substrate on the MS. With all the parameters and conditions optimized for MPS III A DBS assay, a MPS III A assay with previously identified MPS III A patients was conducted. FP blank was used as a negative control; 8 MPS III A patients were tested along with 238 random newborns. The result was summarized in figure 25, seven of 8 MPS-IIIA patients had sulfamidase activity less than 0.02 μmol/L/h, and one patient had an activity of 0.056 μmol/L/h. The random newborns had activity in the range 0.10 – 0.81

μmol/L/h with a mean activity of 0.3 μmol/L/h. The one patient with a 0.056 μmol/L/h activity is less than 20% of the mean activity of the 238 random newborn DBS and it is well below the lowest newborn DBS activity (0.10 μmol/L/h). Thus, all MPS-IIIA patients separate from the large cohort of non-MPS-IIIA newborns.

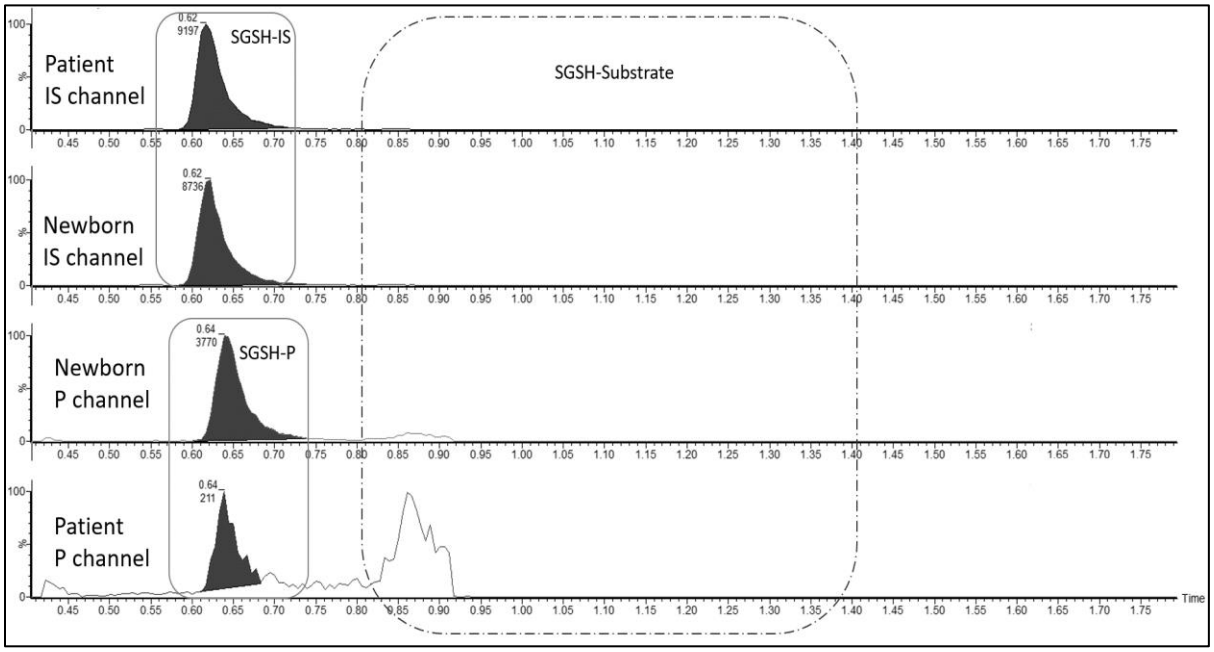
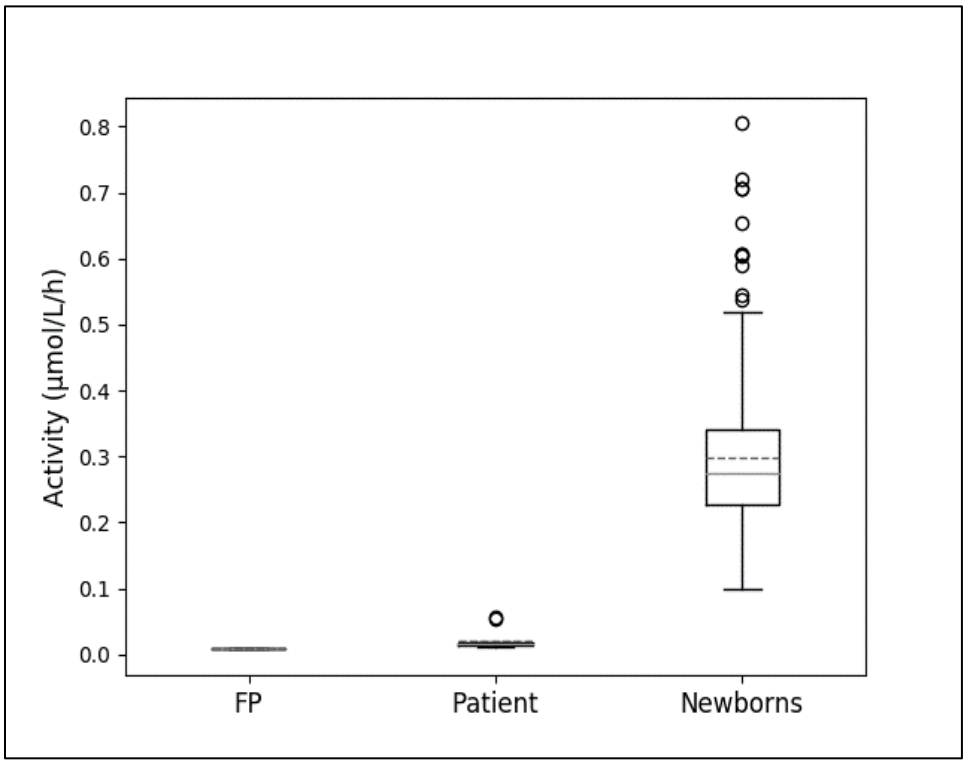


Figure 24. MPS III A assay chromatogram: top two panels are internal standard channels for patient and newborn, respectively; bottom two panels are product channels for newborn and patient, respectively. SGSH-Substrate appears after 0.9 min and peak at 1.22 min. SGSH-IS: m/z 313.2 > 162.1, 0.62 min.; SGSH-P: 306.2 > 162.1, 0.64min.

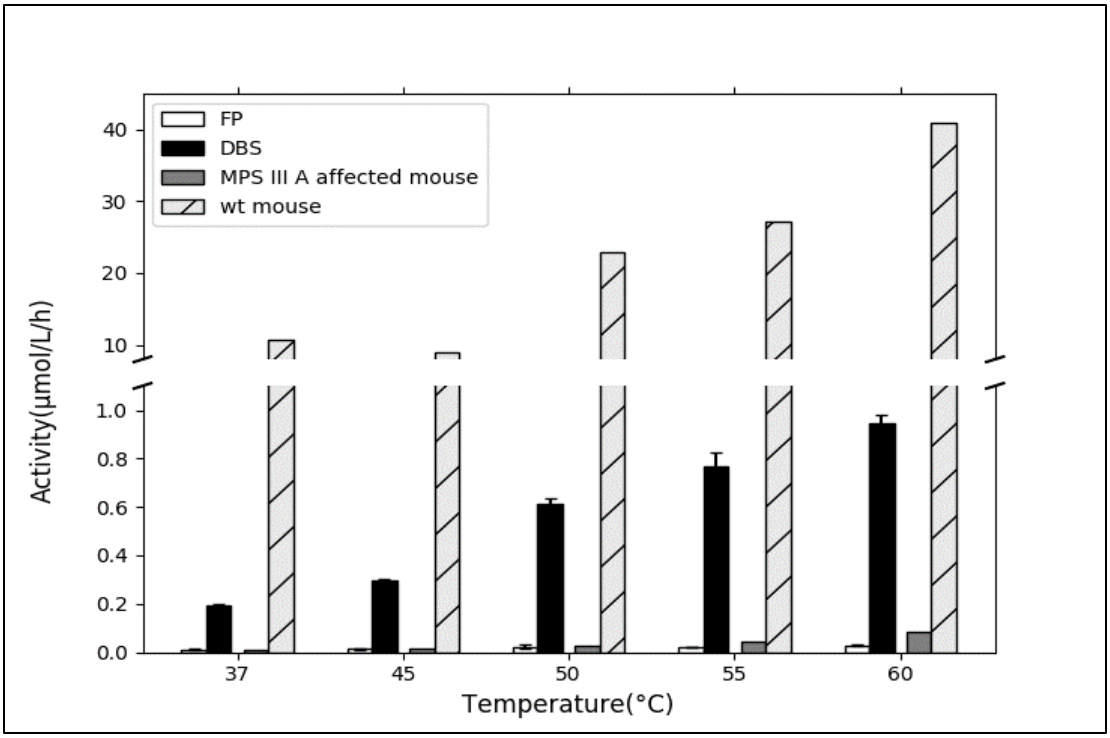
The separation between patient DBS and normal newborn DBS in sulfamidase activity satisfy the requirement for population screening, but a larger cohort of newborns must be tested in a pilot study to establish an accurate cutoff value for MPS III A patient identification as well as validify the usefulness of GlcNS-2-Nap as a screening substrate for MPS III A. Furthermore, the effect of incubation temperature on the MPS III A DBS assay was studied. Five incubation temperature was used: 37, 45, 50, 55 and 60 °C. As shown in Figure 26, sulfamidase has shown excellent thermal stability over the range from 37 to 60 °C. The activity of sulfamidase has a

positive relationship with incubation temperature. As the incubation temperature increases, the activity of the sulfamidase in DBS also increases while the FP blank activity level stays low. The finding could be useful if a more accurate diagnosis needs to be made with elevated activity of sulfamidase from a single DBS punch.



Sample	Mean activity (µmol/L/h)	Activity range(µmol/L/h)	
		lowest	highest
MPS III A patient	0.021	0.013	0.056
Random newborn	0.299	0.100	0.805

Figure 25. MPS III A DBS assay with MPS III A patients and random newborn. seven of the eight MPS-IIIA patients had sulfamidase activity less than 0.02 µmol/L/h, and one patient had an activity of 0.056 µmol/L/h. The random newborns had activity in the range 0.10 – 0.81 µmol/L/h with a mean activity of 0.299 µmol/L/h. The one patient with a 0.056 µmol/L/h activity is less than 20% of the mean activity of the 238 random newborn DBS and it is well below the lowest newborn DBS activity (0.10 µmol/L/h).



	Activity(µmol/L/h)		SD		CV%	
temperature	FP	DBS	FP	DBS	FP	DBS
37	0.0107	0.1916	0.00349	0.0055	32.7	2.8
45	0.0122	0.2974	0.00172	0.0055	14.0	1.8
50	0.0228	0.6108	0.00697	0.0221	30.6	3.6
55	0.0220	0.7681	0.00128	0.0548	5.8	7.1
60	0.0274	0.9444	0.00318	0.0336	11.6	3.6

	activity(µmol/L/h)	
temperature	MPS III A mouse	wt mouse
37	0.0095	10.6972
45	0.0166	9.0705
50	0.0255	22.8142
55	0.0417	27.2168
60	0.0814	40.8611

Figure 26. The effect of incubation temperature on MPS III A DBS assay.

To establish a storing condition for the DBS, an experiment was conducted with three storing temperatures: -20 °C, 4 °C and room temperature. More specifically, fresh prepared DBS was separated in Eppendorf tubes and then put into -20 °C freezer, 4 °C fridge and at room

temperature for a period of time. As summarized in Figure 27, when DBS was stored in a constant temperature, the sulfamidase appeared to be stable over a period of 68 days. Under -20 °C and 4 °C, the sulfamidase shown excellent stability over the 68 days period; while as the sulfamidase activity suffered a sudden drop at 22 days, which was believed to be caused by the raising temperature from a broken AC at that day.

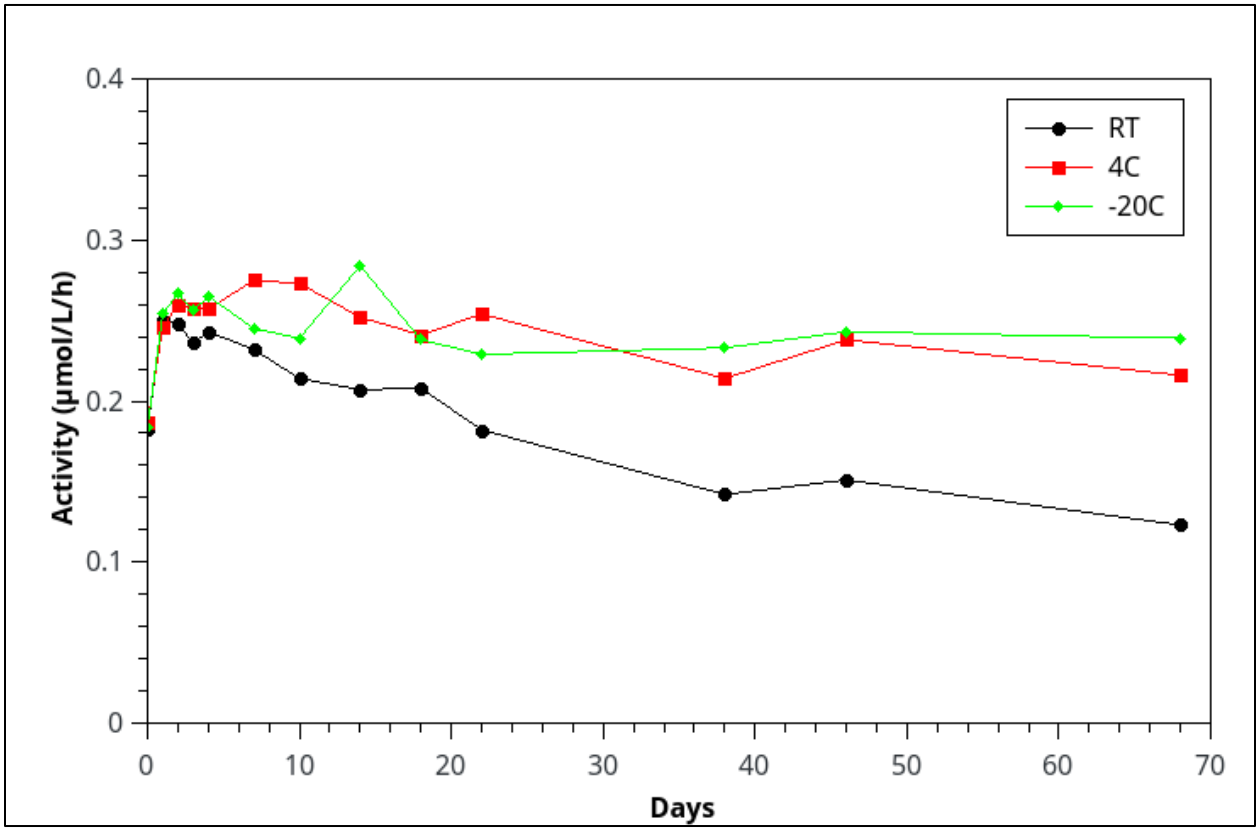
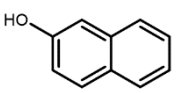
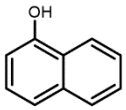
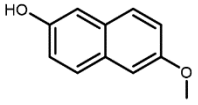
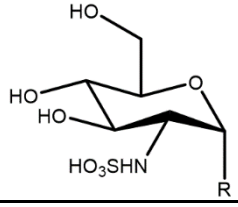
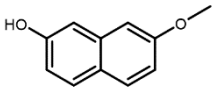
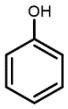
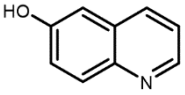
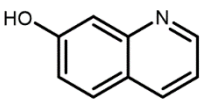
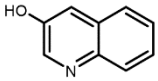
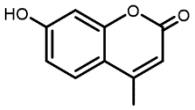
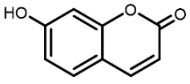


Figure 27. Storing temperature assay with DBS. (blue dot represents -20 °C; orange dot represents 4 °C; and grey dot represents room temperature)

The new substrate for sulfamidase, GlcNS-2-Nap, has shown its potential to be a suitable substrate in a large screening project for MPS III A. Furthermore, it also showed the potential in modifying the aglycone structure for optimizing sulfamidase activity. To investigate if better aglycone structures could be found for sulfamidase, 9 analogs of GlcNS-2-Nap were made

following the synthesis of GlcNS-2-Nap but substitute 2-naphthol with corresponding aromatic structure. As shown in Table 3, the relative MS/MS response for all the analogs in a DBS assay was summarized and it is shown that only one analog, with a 7-hydroxyquinoline aglycone, has the potential of being a good MPS III A assay substrate. However, GlcNS-2-Nap is still the choice of substrate for sulfamidase in MPS III A assay because of the economic reason for the synthesis of the internal standard.

Table 3. Analogs of GlcNS-2-Nap for the sulfamidase substrate.

R group				General structure of sulfamidase substrate 
Relative MS/MS response	1	0.05	0.01	
R group				
Relative MS/MS response	0	0.36	0	1.08
R group				
Relative MS/MS response	0	0.16	0.32	

4. Nonreducing end (NRE) biomarker for MPS III A

4.1. Introduction of NRE method

The target molecule for sulfamidase is called heparan sulfate. It has a diverse structure because of its sulfation and acetylation pattern. In the healthy individual, the natural state of heparan sulfate should be a mixture of all possible structures; however, in a MPS III A patient, all the degradation of heparan sulfate should be stopped at the hydrolysis of 2-sulfate from glucosamine residue in the heparan sulfate chain and results in the same nonreducing end for all possible heparan sulfate structures (Figure 28).

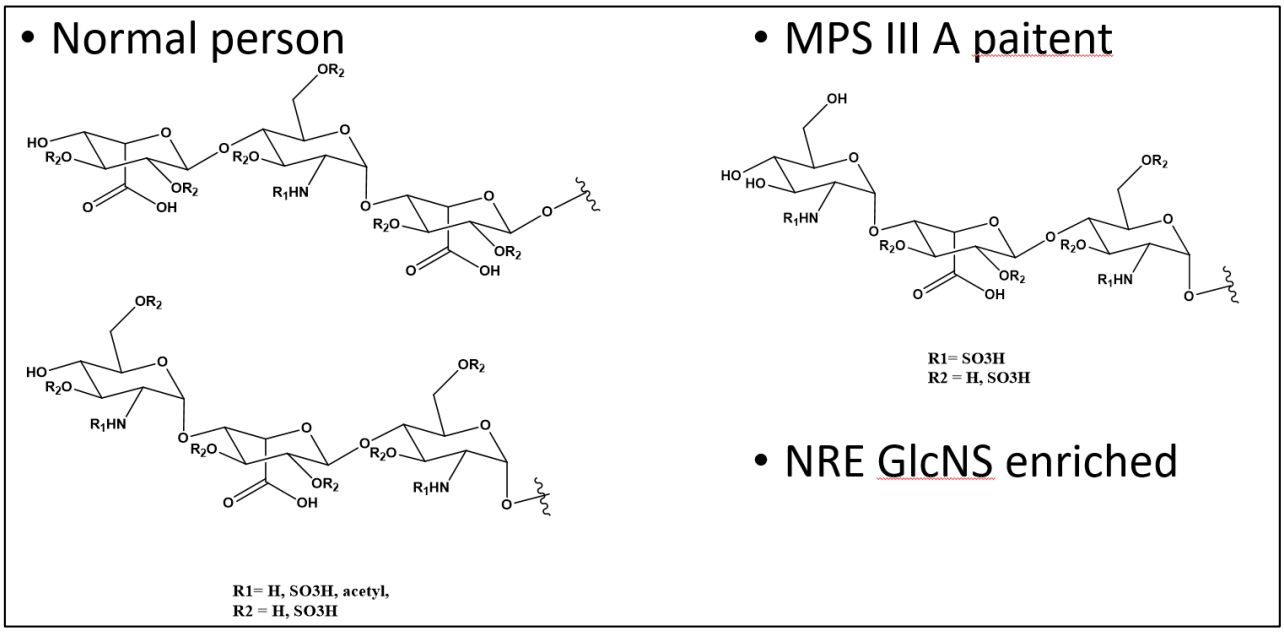


Figure 28. The structural difference between a healthy individual and a MPS III A patient in heparan sulfate

The enrichment of glucosamine-2-N-sulfate in a MPS III A patient provides an opportunity for detecting a specific biomarker for MPS III A. The nonreducing end (NRE) of the polysaccharide chain is a unique glucosamine residue with 2-sulfate attached. None of the other MPS will result in the same NRE. This method has a higher potential for screening than other biomarkers used for MPS III A. One common marker for MPS III A is a disaccharide unit,

D0A0, generated by the action of three heparinase enzymes: heparinase I-III.[36,37] However, this disaccharide biomarker is not specific to MPS III A and it causes a high false positive rate for screening. Before the new MPS III A substrate was discovered, an attempt on developing a biomarker screening method for MPS III A was conducted.

4.2. Nitrous acid degradation for generating NRE biomarker

4.2.1. Introduction to nitrous acid degradation of heparan sulfate

Nitrous acid degradation is a well-established method for generating heparan sulfate fragments.[38–40] At pH 1.5, the N-sulfate on heparan sulfate undergoes hydrolysis and yield a free amine which will then go through a deamination reaction with nitrous acid to give an anhydromannose as the product. There will be no reaction if the amine is acetylated. In this way, the nitrous acid will be selectively cleaving between the glucosamine-N-sulfate and uronic acid residue in heparan sulfate.

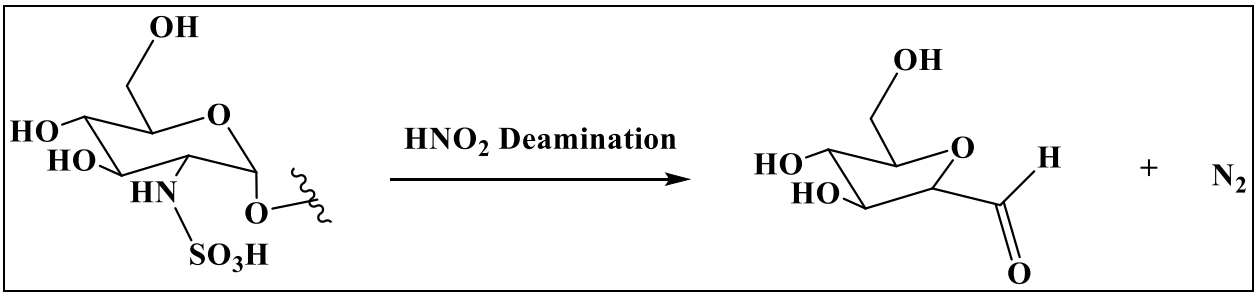


Figure 29. Nitrous acid degradation of heparan sulfate with 2-sulfate NRE

Traditionally, a reductive amination with isotope-labeled sodium borohydride will be performed and yields a deuterium labeled reduced product, which will be used as a tool for quantification.[40]

To incorporate this NRE biomarker with MS, a MS tag must be introduced since there is no ionization site of the anhydromannose product. According to Lawrence and coworkers,[41] aniline could be introduced to the aldehyde via a reductive amination reaction; however, this reaction is done with sodium borohydride in DMSO, which is not an acceptable condition and reagent for a newborn screening lab. Sodium borohydride is toxic and incompatible with water. A new reagent, 2-picoline-borane was developed by Ruhaak and coworkers for reductive amination in water.[42] With the help of this new reagent, a method for reductive amination of the anhydromannose was developed.

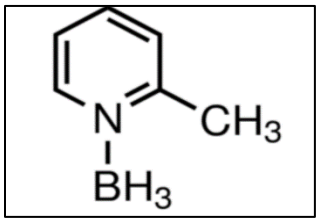


Figure 30. structure of 2-picoline-boran

4.2.2. Method for nitrous acid degradation of heparan sulfate in DBS

To perform a nitrous acid degradation of heparan sulfate in DBS, the following method was executed: DBS was added into 30 uL HNO_2, which was generated *in situ* by mixing 0.5 mM H_2SO_4 and 0.5 mM $Ba(NO_2)_2$, for 30 min, then 5 uL 100 mM aniline and 2 uL 1 M pico-BH_3 were added, reaction was left in the incubator for 2 hours at 37 °C. Then, the reaction was neutralized with NH_4OH. Following the addition of 100uL water, the sample was injected into MS. This method was optimized for the reaction time and concentration of reagent used. The effective reaction yield was above 50% as shown in Table 4. Two groups of reactions were carried out: first, the control group with anhydromannose-aniline standard added after the reaction; secondly, glucosamine-N-sulfate was spiked on to DBS. From the MS response between the two groups and the amount of compound injected to MS, the reaction yield was estimated around 50%. This yield of anhydromannose-aniline is an acceptable number considering the sensitivity of the MS.

Table 4. The yield of nitrous acid degradation reaction with glucosamine N-sulfate spiked DBS

[compound] (uM)	nmol injected	Standard AMA response	GlcNS product response	%yield
1000	320	453770.8	284481.5	62.7
10	3.2	4220.3	2129.1	50.4
1	0.32	664.1	361.7	54.5

4.2.3. Evaluation of anhydromannose-aniline as a biomarker for MPS III A

With the optimized reaction conditions and an acceptable yield of 50%, a series of assays were conducted to evaluate the possibility of using anhydromannose with aniline tag (AMA) as a biomarker for MPS III A. As indicated in Table 5, when a series of dilution of glucosamine-N-sulfate (GlcNS) were spiked onto DBS and subjected to nitrous acid degradation, this NRE method was not able to distinguish below a concentration of 1 μM GlcNS. This concentration is clearly higher than the physiological heparan sulfate concentration in the cell.

Table 5. Nitrous acid degradation of glucosamine-N-sulfate on DBS sensitivity test

Name	pmol injected	AMA TIC
blank DBS	0	2503
100 μM GlcNS DBS	25.6	207125.5
10 μM GlcNS DBS	2.56	17649
1 μM GlcNS DBS	0.256	3175.5
100 nM GlcNS DBS	0.0256	1364.5
10 nM GlcNS DBS	0.00256	1737

Moreover, the presence of AMA signal in the blank DBS, which is a normal DBS without any addition of GlcNS, posed a high background problem. This high background could be the result of nonspecific deamination of other sugars present in the human blood since the nitrous acid degradation is a robust method for all sugar with a similar GlcNS structure within the human cell.

4.2.4. Evaluation of anhydromannose-derivatives as biomarkers for MPS III A

There is no way to circumvent the high background issue with AMA NRE method, so attempts were made to find a viable replacement for the aniline tag. This exploration was conducted under the assumption that the background would not go up as a new tag was introduced to anhydromannose, i.e. the background was caused by non-HS related sugar, otherwise, there was no chance that this method would work. The rationale behind this exploration is that aniline offers a protonation site for AMA; however, it does not offer a dominant fragment from CID. The distribution of fragments from AMA would lower the sensitivity of AMA in SRM mode of the MS. To solve this problem, new tags were tested with the established method mentioned above.

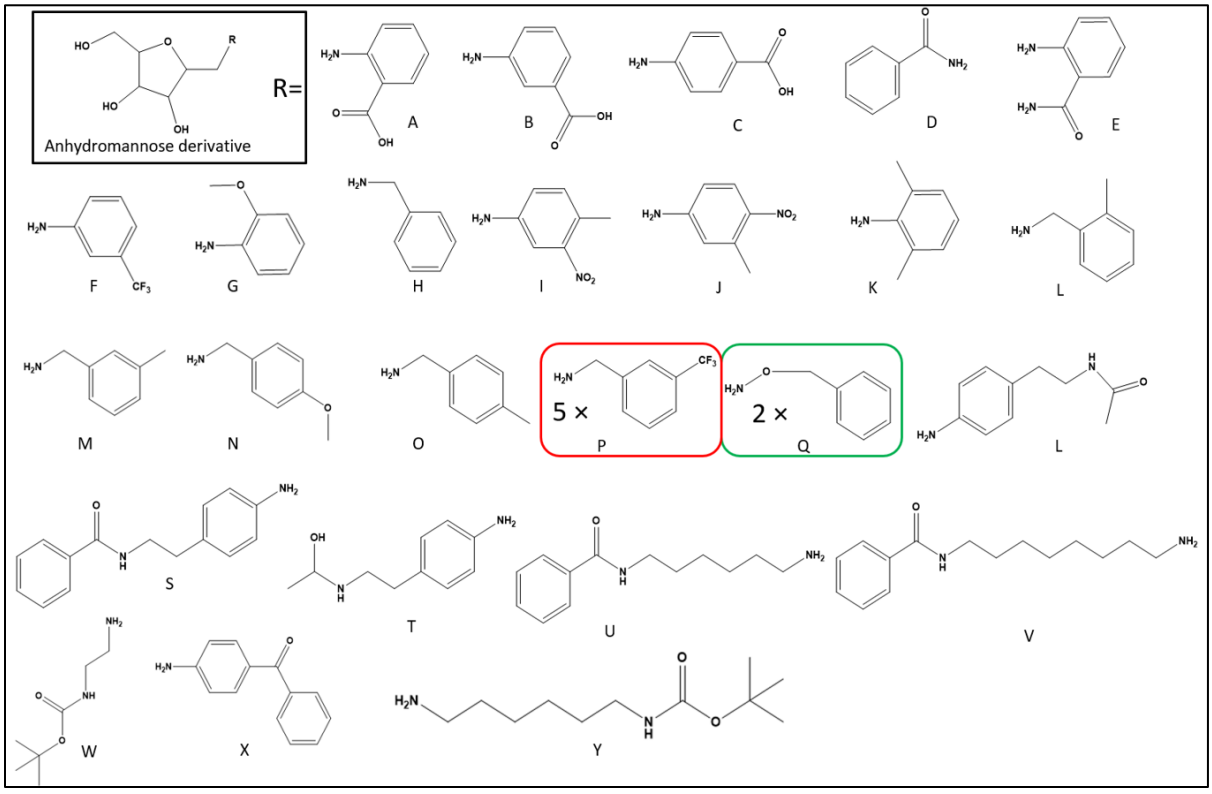


Figure 31. The anhydromannose derivative tried for NRE method

Figure 31 summarized all the anhydromannose derivatives tried for the NRE method. Within the 25 derivatives, two of them showed increased sensitivity in MS: P [3-(trifluoromethyl) benzylamine] and Q [O-enzylhydroxylamine]. However, the new tags did not solve the problem with high background.

This result suggested that the background was contributed by non-heparan sulfate generated anhydromannose. According to Ruijter and coworkers, there was about 3 μM of the most abundant disaccharide biomarker, D0A0, in a patient's DBS.[36] Considering the multiple copies of this D0A0 in one heparan sulfate molecule, the possibility of heparan sulfate has a higher than the 1μM concentration in normal DBS is minimal. Thus, a more specific method of degrading heparan sulfate is required for the NRE method to work.

4.3. Enzymatic degradation of heparan sulfate for generating NRE biomarker

4.3.1. Introduction to heparinase I-III degradation of heparan sulfate

Heparinase I-III are three enzymes that are used in the degradation of heparan sulfate in the literature. They are endoglycosidases that will cleave heparan sulfate between the glucosamine and uronic acid residue. In the MPS III A patient, this will result in an increased level of NRE residue, glucosamine-N-sulfate, as it is enriched by the nature of this disease. The three enzyme each has a different preference for the degree of sulfation and acetylation, so it is safer to use all three of the heparinase for a complete degradation of the heparan sulfate.[39,43]

4.3.2. Method for heparinase degradation of heparan sulfate in DBS

The heparinase degradation of heparan sulfate in DBS was carried out in the following buffer conditions: 40 mM ammonium acetate, 3.33 mM of calcium acetate in milliQ-water at pH 7.0. In a 96 deep-well plate, 0.5 mU of each heparinase was used per assay with 50 µL assay buffer and 3 DBS punches.

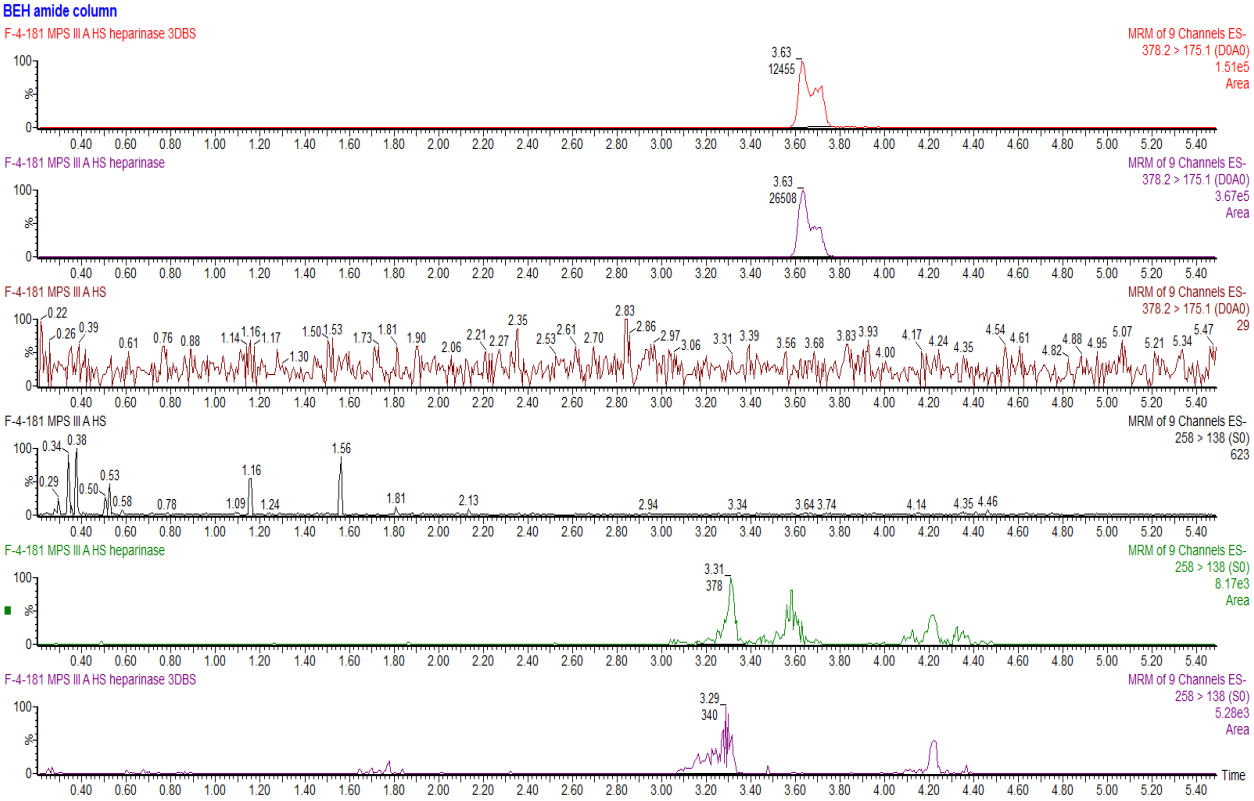


Figure 32. Chromatograms for heparinase degradation of heparan sulfate. (from top to bottom: D0A0 channel for heparinase assay with 2 µg of heparan sulfate in 3 DBS; D0A0 channel for heparinase assay with 2 µg of heparan sulfate without DBS; D0A0 channel with 2 µg of heparan sulfate; S0 channel with 2 µg of heparan sulfate; S0 channel for heparinase assay with 2 µg of heparan sulfate without DBS; S0 channel for heparinase assay with 2 µg of heparan sulfate in 3 DBS)

4.3.3. Evaluation of heparinase degradation NRE as a biomarker for MPS III A

To evaluate the possibility of using heparinase to generate NRE biomarker for MPS III A, heparan sulfate was purchase from Sigma (cat. # H7640-10MG). 2 µg of HS was subjected to

three conditions: 1. No heparinase and DBS; 2. With 0.5 mU of each heparinase but no DBS; 3. With 0.5 mU of each heparinase and 3 DBS. Two markers were observed: S0 and D0A0. As Figure 32 shows, under condition 2 and 3, there were corresponding S0 and D0A0 and there was a 2-fold signal suppression from DBS sample. Under condition 1, there was no marker generated. However, the signal from S0 was 36 times less than the one from D0A0 as there is more D0A0 in one HS molecule. With 2 µg of HS, only 340 counts for S0 was observed.

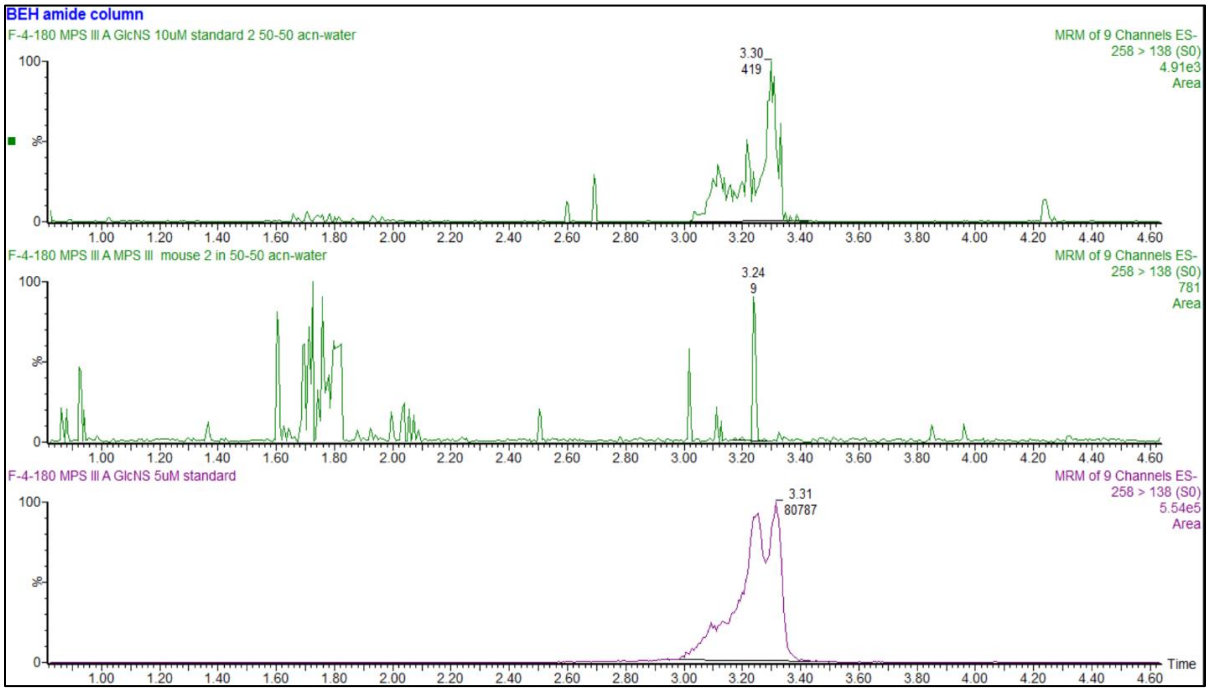


Figure 33. Chromatograms for MPS III A mouse DBS. (from top to bottom: S0 in MPS III A mouse; S0 in wild type mouse; and S0 standard (5 µM).

MPS III A affected mouse and wild-type mouse DBS were obtained from Biomarin Inc.. 4 mouse DBS punches were used in the assay and Waters Xevo was used for S0 detection. As shown in Figure 33, there was almost no signal from S0 with the wild-type mouse; on the other hand, an ion count of 419 for S0 was observed with the MPS III A affected mouse DBS. The result suggested that heparinase degradation of HS could yield specific biomarker S0 for MPS III A. There is no high background issue as nitrous acid method because of the specific action of

heparinase towards heparan sulfate. However, there are several drawbacks for the NRE method: 1. The runtime for each injection is 5.5 min, which is considered too long for a screening process; 2. 4 DBS punches were used per assay. 3. The requirement of top-tier MS for an adequate sensitivity.

Consider all these drawbacks for NRE methods, they are not continued for the MPS III A project. Enzymatic reaction with GlcNS-2-Nap is obviously the better choice.

5. Synthesis of compound involved in MPS III A assay

1, 3, 4, 6-Tetra-*O*-acetyl-2-azido-2-deoxy-α/β-D-glucopyranoside (3).

Preparation of TfN_3: To a solution of NaN_3 (23 g, 354 mmol) in H_2O (60 mL) was added 100 mL of DCM at 0 °C. The resulting biphasic mixture was stirred vigorously and Tf_2O (12 mL, 70 mmol) was added slowly over a period of about 10 min. The reaction mixture was in ice bath for 2 h. The organic phase was separated, and the aqueous phase was extracted with DCM (50 mL x 2). The organic layer was washed with saturated $NaHCO_3$ solution and used for next step without further purification. (***Caution: TfN_3 is explosive when concentrated to dryness. Use appropriate facial mask and shield all the time.)***

Glucosamine hydrochloride (7.64 g, 35.4 mmol) was dissolved in H_2O (100 mL) and treated with potassium carbonate (7.34 g, 276.4 mmol) and $CuSO_4$ hydrate (88 mg, 0.35 mmol). To the reaction mixture was added the above mentioned TfN_3 solution. Then, MeOH (200 mL +) was added until the solution was homogeneous. The reaction was allowed to stir for 18 h at room temperature and the solvent was removed under reduced pressure. The residue was dissolved in pyridine (150 mL); to it, catalytic amount of DMAP and acetic anhydride (50 mL, 45.3 mmol) were added at 0 °C and the reaction mixture was stirred for overnight at room temperature. Upon

completion of reaction, MeOH (50 mL) was added and the reaction mixture was stirred for 10 min. The solvent was evaporated at strictly below 40 °C. The resulted residue was diluted with EtOAc (250 mL) and washed with water, 10% $CuSO_4$ in H2O, saturated $NaHCO_3$ and saturated NaCl. The organic layer was dried over $NaSO_4$ and concentrated by rotary evaporation. The residue was purified by silica gel column chromatography (0-50% EtOAc in Hexane) to afford product **3** (mixture of α/β) (12.2 g, 32.7mmol) in 92 % yield. 1H NMR (300 MHz, CDCl3) (β isomer): δ 5.54 (d, J = 8.6 Hz, 1H), 5.14 – 4.97 (m, 2H), 4.34 – 4.23 (m, 1H), 4.06 (d, J = 11.9 Hz, 1H), 3.79 (dd, J = 7.5, 2.0 Hz, 1H), 3.65 (t, J = 8.3 Hz, 1H) 2.18 (s, 3H), 2.08 (s, 3H), 2.06 (s, 3H), 1.97 (s, 3H). MS *m/z* 396.6 $[M+Na]^+$.

3, 4, 6-Tri-*O*-acetyl-2-azido-2-deoxy- α/β -D-glucopyranoside (4). To a solution of compound **3** (12.2 g, 32.7 mmol) in dry DMF (90 mL) under a nitrogen atmosphere was added hydrazine acetate (4.5 g, 49 mmol) at 0 °C. The reaction mixture was stirred for 30 min (extended time may result in the production of byproduct) at room temperature. Then, the reaction was diluted with ethyl acetate and washed with saturated sodium bicarbonate, water, and brine. Afterward, the mixture was dried over sodium sulfate, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography (0- 50% EtOAc in Hexane) to give product **4** (9.3 g, 28.1 mmol) in 85.8% yield. MS *m/z* 354.1 $[M+Na]^+$.

3, 4, 6-Tri-*O*-acetyl-2-azido-2-deoxy-1-fluoro- α/β -D-glucopyranoside (5). Compound **4** (9.3 g, 28.1 mmol) was dissolved in dry THF (60 mL) and Diethylaminosulfur trifluoride (DAST) (5.5 mL, 42.1 mmol) was added to the stirred solution at -30 °C under a nitrogen atmosphere. After addition of DAST, the ice bath was removed, and the solution was allowed to warm up to

room temperature and stirred for overnight. The reaction was quenched with MeOH (20 mL) and concentrated under reduced pressure. Column chromatography on silica gel (0-50% EtOAc in Hexane) afforded **5** (8.0 g, 24 mmol) in 85.4% yield. MS *m/z* 356.1 $[M+Na]^+$.

N-(6-Amino-hexyl)-acetamide (6). To methyl acetate (122.4 mmol), hexane- 1,6-diamine (14.2 g, 122.4 mmol) and water (6.2 mL) were added and the mixture was heated to 100°C for 24 hours under constant stirring. The reaction mixture was cooled to room temperature and directly loaded on to a short silica column. Upon elution with 10 to 20% of methanol (with 10% NH4OH) in CH2Cl2, the desired mono-acetated product **6** was obtained (10.5 g) in 49.7% yield.

Pentanoic acid [2-(4-hydroxy-phenylcarbamoyl)-ethyl]-(6-acetamino-hexyl)-amide (8). 4-Acrylamido-phenol **7** (9.46 g, 58 mmol) and mono-propionyl-1,6-hexanediamine **6** (10.5 g) were dissolved in a solution of isopropanol (450 mL) and water (50 mL) and heated in an oil bath at 65 °C for 48 hrs. The reaction mixture was concentrated by rotary evaporation to afford the Michael addition product, which was used for the next step without further purification. To the residue from the above step was added CH2Cl2 (150 mL), DMF (15 mL) and 150 mL of saturated sodium bicarbonate in water. Pentanoyl chloride (7.96 mL, 67.1 mmol) was added dropwise at room temperature with stirring, and the mixture was stirred for an additional 6 h at room temperature. The organic layer was separated, and the water layer was extracted twice with 150 mL portions of 5% MeOH in CH2Cl2. The organic layers were combined and concentrated by rotary evaporation. The residue was purified by silica gel column chromatography (1-15% MeOH in CH2Cl2) to afford MPS –III A aglycone **8** (18.6 g) in 76.4 % yield. MS *m/z* 406.3 $[M+H]^+$.

Acetic acid 3-acetoxy-2-acetoxymethyl-5-azido-6-(4-{3-[pentanoyl-(6-acetamino-hexyl)-amino]-propionylamino}-phenoxy)-tetrahydro-pyran-4-yl ester (9). MPS-III A aglycone **8** (13.5 mmol, 1.1eq), 3, 4, 6-Tri-*O*-acetyl-2-azido-2-deoxy-1-fluoro- α/β -D-glucopyranoside **5** (4.1 g, 12.3 mmol, 1 eq) and 2,6-di-*tert*-butyl-4-methylpyridine (5.05 g, 24.6 mmol, 2 eq) were dried for 1 hr under high vacuum (oil pump) and dissolved in dry CH_2Cl_2 (615 mL, 0.02 M). BF3.Et2O (12.4 mL, 98.4 mmol, 8 eq) was added dropwise with stirring at room temperature under a nitrogen atmosphere. After the reaction mixture had been stirred for 2.5 h at room temperature, 350 mL of saturated aqueous NaHCO3 was added. The aqueous layer was extracted with CH2Cl2 and the organic extracts were combined and washed with water, brine and dried over anhydrous Na2SO4. The solution was filtered and concentrated by rotary evaporation. The residue was purified by silica gel column chromatography (CH2Cl2, then 1-10% MeOH in CH2Cl2) to afford product **9** (3.5 g, 4.77 mmol) in 39% yield. MS *m/z* 719.3 $[M+H]^+$.

Acetic acid 4-acetoxy-2-acetoxymethyl-5-amino-6-(4-{3-[pentanoyl-(6-acetamino-hexyl)-amino]-propionylamino}-phenoxy)-tetrahydro-pyran-3-yl ester (10). Compound **9** (4.77 mmol, 1 eq) was dissolved in dry acetonitrile (24 mL) under nitrogen atmosphere and was cooled in an ice bath. To this solution was added an ice-cold solution of tin(II) chloride (181 mg, 0.95 mmol, 0.20 eq), triethylamine (2 mL, 14.3 mmol, 3eq) and thiophenol (1.95 mL, 19 mmol, 4 eq) in 15 mL of dry acetonitrile. The solution was stirred at 0 °C for 2 hours and concentrated by rotary evaporation, diluted with dichloromethane then washed with cold 1N NaOH, water and brine. The organic layer was separated and concentrated by rotary evaporation, and the crude product was used for the next step without further purification. MS *m/z* 693.3 $[M+H]^+$.

Acetic acid 4-acetoxy-2-acetoxymethyl-6-(4-{3-[pentanoyl-(6-acetamino-hexyl)- amino]-propionylamino}-phenoxy)-5-(2,2,2-trifluoro-acetylamino)-tetrahydro-pyran-3-yl ester (11). The crude product **10** was dried under high vacuum and dissolved in dry of dichloro-methane (20 mL) and pyridine (3.84 mL, 47.7 mmol, 10 eq) was first added, followed by drop-wise addition of trifluoroacetic anhydride (1 mL, 7.15 mmol, 1.5 eq). the resulted mixture was stirred for 4h at room temperature under nitrogen atmosphere. The solvent was removed by ro-tary evaporation, and the residue was purified using flash chromatography with 1-5% MeOH in DCM to give product **11** (α:β:57:43) (2.5 g) in 65% yield. 1H NMR (300 MHz, CDCl3) (α iso-mer): δ 7.54 (d, *J* = 9.0 Hz, 2H), 7.02 (d, *J* = 9.0 Hz, 2H), 5.57 (d, *J* = 3.5 Hz, 1H), 5.49(t, *J* = 9.6 Hz, 1H), 5.22 (t, *J* = 9.7 Hz, 1H), 4.52 – 4.41 (m, 1H), 4.26 (dd, *J* = 12.1, 4.4 Hz, 1H), 4.16 – 4.02 (m, 2H), 3.68 (t, J = 6.4 Hz, 3H), 3.32 – 3.19 (m, 4H), 2.66 (t, *J* = 6.4 Hz, 2H), 2.34 – 2.27 (m, 2H), 2.24 – 2.14 (m, 2H), 2.10 – 2.01 (m, 9H), 1.68 – 1.28 (m, 12H), 0.90 (t, *J* = 7.3 Hz, 3H). MS *m/z* 789.3 [M+Na]+.

Compound (12). compound **11** (109.7 mg) was dissolved in 6 mL 7 N ammonia in methanol and 2.2 mL of 2 M ammonium water was added to the reaction. Reaction was then sealed and left at room temperature overnight. Next day, the reaction was dried and purified by HPLC with C18 column to give product 12 (35.9 mg, 45.5%). MS *m/z* 567.3 [M+Na]+.

Compound (13). The crude compound **12** (35.9 mg, 0.064 mmol) was dissolved in 102 mL wa-ter and sulfur trioxide pyridine complex (10 eq, 0.1 g) was added to the solution in portions. Then, the pH of the reaction was adjusted by adding 4 M aqueous NaOH to about 8.5-9. The

color change could be a good indicator of the pH. The reaction started out as a light-yellow solution, and it turned into a dark red color when the pH was adjusted to around 9. The pH needs to be checked every 10 minutes until it was stable and then the reaction was left for 1 day until completion. Another 5 eq of sulfur trioxide pyridine could be added if the reaction did not reach completion after 1 day. Upon completion, the reaction was filtered and purified by HPLC with C18 column to obtain compound **13** with a 52.3% yield (21.5 mg).

^{1}H NMR (300 MHz, MeOD) δ 7.51 – 7.44 (m, 2H), 7.16 (dd, *J* = 8.9, 2.0 Hz, 2H), 5.76 (d, *J* = 3.3 Hz, 1H), 3.84 – 3.63 (m, 6H), 3.57 – 3.34 (m, 4H), 3.15 (t, *J* = 6.8 Hz, 2H), 2.64 (dd, *J* = 15.7, 7.0 Hz, 2H), 2.50 – 2.31 (m, 2H), 1.94 (s, 3H), 1.69 – 1.25 (m, 12H), 0.94 (td, *J* = 7.3, 2.6 Hz, 3H). MS *m/z* 645.3 $[M]^-$.

Compound (18). Compound **4** (233.5 mg, 0.705 mmol) was dissolved in 5 mL dry DCM. Then, anhydrous K2CO3 (2g) was added to the solution. Trichloroacetonitrile (0.424 mL, 6 eq) was then added to the reaction in a dropwise fashion. The reaction was allowed to stir for 2 days under nitrogen. Upon completion, the reaction was cooled to 0 °C and then diluted with DCM and quenched with ice cold water. The aqueous layer was extracted with DCM then the combined organic layer was washed with brine and dried over sodium sulfate. The solvent was removed. Then the product was purified with a silica column (0-2.5% MeOH in DCM with 0.1 M ammonium water). The product is not stable on silica gel under acidic condition. The column must be prepared with the basic condition. Compound **18** was obtained as a mixture of alpha and beta isomers with a 64.6% yield (216.5mg).

^{1}H NMR (300 MHz, $CDCl_3$) δ 8.83 (s, 1H), 6.49 (d, *J* = 3.6 Hz, 1H), 5.56 – 5.47 (m, 1H), 5.15 (t, *J* = 9.7 Hz, 1H), 4.33 – 4.06 (m, 4H), 3.77 (dd, *J* = 10.5, 3.6 Hz, 1H), 2.14 – 2.01 (m, 12H). MS *m/z* 476.3 $[M+H]^+$.

Compound (**20**). Diacetone-D-glucose (20g g, 77 mmol) was dissolved in 50 mL dry DMF with tetrabutylammonium iodide (2.84g 0.1 eq). Then, NaH (4.6g, 1.5 eq) was added to the solution slowly in an ice bath. The reaction was allowed to stir for 1 hour under nitrogen. Then, benzylbromide (10.97 mL, 1.2 eq) was added to the reaction dropwise. The reaction was left overnight. Upon completion, the reaction was quenched with 50 mL MeOH. The solvent was removed and the residue was redissolved in DCM and then washed with brine. After dried with MgSO4, the crude product was dried and subjected to the next step without further purification. MS m/z 373.62 $[M+Na]^+$.

Compound (**21**). Compound **20** was dissolved in 200 mL 80/20 acidic acid/water. Then, the reaction was warmed to 60 °C and stirred for 1 hour. Then, the reaction was concentrated and co-evaporated with toluene and subjected to the next step without further purification. MS *m/z* 333.3 $[M+Na]^+$.

Compound (**22**). Compound **21** was dissolved in 125 mL anhydrous pyridine and 63 mL DCM. The solution was placed in an ice bath and to it, pivaloyl chloride (1.5 eq) was added. The mixture was allowed to react at RT for 3.5 hours and then was cooled to 0 °C. 20 mL of MeOH was added to quench the reaction. Then, the solution was diluted with DCM (70 mL). The organic layer was washed with sodium bicarbonate, water, and brine. After dried over MgSO4,

the solvent was removed and the final product compound **22** (16.5 g, 41.8 mmole) was obtained after recrystallization with hexane as a white powder with a 54% yield over three steps. MS m/z 433.6 $[M+K]^+$.

Compound (**24**). Compound **22** (11 mg, 28 mmol) was dissolved in 150 mL anhydrous dichloroethane and 12 mL anhydrous pyridine. To it, triflic anhydride was added dropwise at -15 °C. The reaction was stirred at -15 °C for 2 hours. Water (15 mL) was added to the reaction and then, it was allowed to stir at 85 °C for 30 minutes. Then, the reaction was allowed to cool down and diluted with DCM. The organic layer was then washed with cold 1 M HCl, sodium bicarbonate, and brine. It was subjected to next step without further purification. MS m/z 394.5 [M+H]+.

Compound (**25**). Compound **24** was dissolved in anhydrous MeOH and to it, sodium methoxide was added until a pH of 13 was reached. The reaction was left stirring overnight at RT. AcOH was added to neutralize the reaction and the solvent was removed. Residue diluted with DCM and then washed with sodium bicarbonate, water, and brine. The mixture was passed through MgSO4 and the concentrated. MS m/z 311.3 $[M+H]^+$.

Compound (**26**). Crude compound **25** (8.65 g) was dissolved in 80 mL dioxane and 150 mL 0.1 M sulfuric acid was added to it. The mixture was stirred for 5 hours at 80 °C and then was cooled before neutralized with amberlite IRA-400 (OH) resin. The mixture was filtered and concentrated. MS m/z 271.3 $[M+H]^+$.

Compound (**28**). Crude compound **26** (7.52 g) was dissolved in pyridine (100 mL). To it, acetic anhydride (5 eq, 13.2 mL) was added and the reaction was left overnight. The reaction was quenched with water and washed with brine before dried over MgSO4. The crude product was concentrated. MS m/z 439.3 $[M+H]^+$.

Compound (**30**). Crude compound **28** (12.2 g) was dissolved in 50 mL anhydrous DCM. To it, 7.5 mL borontrifluoride diethyletherate (2.2 eq) was added dropwise after the addition of 2.25 mL (1.1 eq) ethanethiol at 0 °C. The reaction was stirred for 4 hours at RT. Sodium bicarbonate was added to quench the reaction. The organic layer was then washed with water and dried over MgSO4. The solvent was removed to afford crude product **30.** MS m/z 441.3 $[M+H]^+$.

Compound (**32**). Crude compound **30** (611 mg) was dissolved in 3.5 mL anhydrous MeOH. To it, a catalytic amount of sodium methoxide was added, and the reaction was left for 1 hour at RT. Then, the reaction was quenched with acidic resin and then filtered. The solvent was removed and carried to next step without further purification. MS m/z 315.1 $[M+H]^+$.

Compound (**34**). Crude compound **32** (425 mg) was dissolved in 3.5 mL acetonitrile. To it, benzaldehyde dimethyl acetal (0.21 mL, 1.5 eq) and camphorsulfonic acid (47 mg, 0.15 eq) were added. The reaction was allowed to stir for 1 hour at RT and then quenched with triethylamine. The solvent was removed by rotovap. The residue war redissolved in DCM and washed with

water and brine. After dried over MgSO4, the crude product **34** was obtained with no further purification. MS m/z 403.3 $[M+H]^+$.

Compound (**35**). Crude compound **34** (540 mg) was dissolved in 4.5mL anhydrous pyridine. To it, acetic anhydride was added at 0 °C. The reaction was left overnight at RT and then quenched with MeOH the next day. The solvent was removed by rotovap and the residue was redissolved in DCM. The DCM solution was then washed with sodium bicarbonate, water and brine. After dried over MgSO4, the crude product **35** was obtained with no further purification. MS m/z 444.1 $[M+H]^+$.

Compound (**38**). Ethanolamine (3 mL, 49.7 mmole) was dissolved in 50 mL anhydrous pyridine. To it, 40 mL DCM solution of benzyl chloroformate (7.8 mL, 1.1 eq) was added slowly at 0 °C. The reaction was left stirring for 2 hours at 0 °C and allowed to warm to RT overnight. Next day, the reaction mixture was washed with sodium bicarbonate, water, and brine, and then dried over MgSO4. The solvent was removed by rotovap and the residue was purified by recrystallization with EA and hexane. 4.23 g of white crystal compound **38** was obtained. MS m/z 195.2 $[M+H]^+$.

Compound (**39**). Crude compound **35** (75 mg), compound **38** (39.5 mg, 1.2 eq) and 4 Å molecular sieve was dried under high vacuum overnight and dissolved in 2.5mL anhydrous DCM the next day. The mixture was then cooled to 0 °C and let stirred for 15 minutes. Then, light-sensitive N-iodosuccinimide (76 mg, 2 eq) was added and followed by the addition of triflic acid (15 µL, 0.1 eq). The reaction was allowed to stir for 30 minutes at RT before quenched by

the addition of triethylamine. The mixture was diluted by DCM and then filtered through celite. The filtrate was washed with 10% sodium thiosulfate, sodium bicarbonate, and water. After dried over MgSO4, the solvent was removed. Crude product compound **39** was obtained with no further purification. MS m/z 600.7 $[M+Na]^+$.

Compound (**40**). Crude compound **39** (97 mg) was dissolved in 3 mL of a solvent mixture, which is composed of 60/2/1 in the volume of DCM/TFA/water at RT. The reaction was left for 4 hours at RT. Then, it was treated with cold sodium bicarbonate. The organic layer was washed with water and dried over MgSO4. The crude product compound **40** was obtained after the solvent was removed by rotovap with no further purification. MS m/z 490.3 $[M+H]^+$.

Compound (**41**). Crude compound **40** (80 mg) was dissolved in 3 mL of a solvent mixture, which is composed of 2/1 in the volume of DCM/water at RT. TEMPO (5 mg, 0.2 eq) and bis(acetoxy)iodobenzene (BAIB) (131 mg, 2.5 eq) were added to the mixture. The reaction was left for 2 hours at RT before quenched with 10% sodium thiosulfate. The organic layer was dried over MgSO4 and solvent was removed by rotovap. The residue was dissolved in 7 mL DMF and to it, cesium carbonate (213 mg, 4 eq) and methyl iodide (15.3 uL, 1.5 eq) were added. The reaction was allowed to stir at RT for 2 hours and then diluted with DCM. The organic layer was washed with water and dried with MgSO4. Then, the residue was purified with a silica column (0-50 % EA in hexane). 51 mg of Compound **41** was obtained with a 60 % yield over 4 steps. MS m/z 540.7 $[M+Na]^+$.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.31 (dd, J = 29.3, 8.8 Hz, 14H), 5.10 (s, 4H), 4.98 (d, J = 8.3 Hz, 4H), 4.81 (s, 2H), 4.76 – 4.60 (m, 4H), 4.07 (s, 2H), 3.82 (d, J = 8.7 Hz, 7H), 3.74 (s, 2H), 3.62 – 3.55 (m, 2H), 3.42 (d, J = 4.8 Hz, 4H), 2.10 (s, 6H).

Compound (**45**). Compound **41** (133 mg, 0.257 mmole) and compound **18** (147 mg, 1.2 eq) were dissolved in 2.5 mL anhydrous DCM with 4Å molecular sieve. The mixture was then cooled to -20 °C. Triflic acid (22.7 uL, 1 eq) was added and the reaction was allowed to stir at -20 °C for 2 hours. Upon completion, the mixture was filtered through celite, and the filtrate was diluted with DCM. DCM solution was then washed with sodium bicarbonate and dried over MgSO4. The solvent was removed, and the residue was subjected to a silica column with 0-80% EA in hexane. 145 mg of compound **45** was obtained with a 68% yield**.** MS m/z 853.8 $[M+Na]^+$.

Compound (**47**). Hexane diamine (2 g, 17.2 mmole) and methyl acetate (1.39 mL, 1 eq) were dissolved in 2 mL of water. The mixture was then heated to 100 °C for 24 hours. The reaction was cooled to RT and columned with silica gel. (200 mL of 10% MeOH/DCM with 2 mL NH4OH). 1.5 g of compound **47** was obtained with 55% yield. MS m/z 159.2 $[M+H]^+$.

^{1}H NMR (300 MHz, MeOD) δ 3.15 (t, J = 7.0 Hz, 2H), 2.68 – 2.57 (m, 2H), 1.92 (s, 3H), 1.57 – 1.28 (m, 8H).

Compound (**48**). Compound **47** (265.5 mg, 1.68 mmole) and methyl acrylate (0.166 mL, 1.0 eq) were dissolved in 5 mL MeOH. Reaction was left for 4 hours. Dried solvent and silica column

was used to purify the product (0-5% MeOH in DCM). 197.2 mg of compound **48** was obtained with 48% yield. MS m/z 245.3 $[M+H]^+$.

^{1}H NMR (300 MHz, MeOD) δ 3.69 (s, 1H), 3.17 (t, J = 7.0 Hz, 1H), 2.85 (t, J = 6.8 Hz, 1H), 2.57 (dt, J = 12.4, 7.0 Hz, 2H), 1.94 (s, 1H), 1.62 – 1.28 (m, 4H).

Compound (**49**). Compound **48** (185.4 mg, 0.76 mmole) was dissolved in 10 mL anhydrous DMF. To it, pentanoyl chloride (0.18 mL, 2.0 eq) was added at 0 °C, dropwise. The reaction was left for 4 hours at RT. The reaction was quenched with addition of sodium bicarbonate and extracted with DCM. The solvent was removed, and the crude product was carried to next step without further purification. MS m/z 328.2 $[M+H]^+$.

Compound (**50**). Compound **49** was dissolved in 15 mL 1 M NaOH. Reaction was left for 30 minutes at RT. The reaction was acidified by HCl. The mixture was extracted three times with ethyl acetate. Then, the organic layer was dried. 83 mg of compound 50 was obtained with 35% yield over 2 steps. MS m/z 315.3 $[M+H]^+$.

^{1}H NMR (300 MHz, MeOD) δ 3.61 (dt, J = 25.4, 7.1 Hz, 5H), 3.44 – 3.26 (m, 6H), 3.22 – 3.09 (m, 5H), 2.58 (dt, J = 14.0, 7.1 Hz, 5H), 2.36 (tt, J = 20.8, 7.4 Hz, 6H), 1.93 (s, 7H), 1.46 (dddd, J = 25.6, 13.6, 13.2, 5.7 Hz, 34H), 0.95 (t, J = 7.2 Hz, 9H).

Compound (**51**). Compound **50** (83 mg, 0.277 mmole) was dissolved in 1.5 mL anhydrous THF. To it, N,N'-Dicyclohexylcarbodiimide (DCC) (1.2 eq, 68.5 mg) was added and the mixture was stirred for 10 minutes. Then, N-hydroxysuccinimide (1.2 eq, 38.2 mg) was added to the mixture

and reaction was stirred for 3 hours at RT under N2. Silica column was used to purify the product (0-5% MeOH in DCM). 86.79 mg of compound **51** was obtained with 76% yield. MS m/z 412.2 $[M+H]^+$.

Compound (**52**). Compound **45** (145 mg, 0.175 mmole) was dissolved in 8.73 mL THF. To it, 1.36 mL 30% H2O2 and 8.73 mL 1 M LiOH were added. The reaction was left overnight at RT. 4 N NaOH was used to change the pH to 14 and stirred for 1 hour. Upon completion, the reaction was neutralized with AcOH and concentrated. The residue was vortexed with water and applied to RP-C18 column. The product was then passed through a column of cation exchange resin Na+ form to obtain 86.8 mg of the sodium form of compound **52** with a 74 % yield**.** MS m/z 646.6 $[M]^-$.

Compound (**54**). Compound **52** (86.8 mg, 0.129 mmole) was dissolved in 10 mL THF. To it, 1 mL of 1M PMe3 in THF and 13 mL 0.1 M NaOH were added. The mixture was allowed to stir at RT for 1 hour. pH was adjusted to 8 by AcOH and then, the mixture was concentrated. RP-C18 column was used to purify the product. 67 mg of compound **54** was obtained with 80% yield. MS m/z 667.7 $[M+Na]^+$.

Compound (**56**). Compound **54** (67mg, 0.0806 mmole) was dissolved in 13 mL MeOH. To it, sulfur trioxide pyridine complex (134 mg, 10 eq) and the mixture of (0.3 mL/0.04 mmol) 1.2 mL Et3N and 3.5 mL 0.1 M NaOH was added at 0 °C. the reaction was left overnight at RT. The solvent was removed and then passed through a short column of cation exchange resin Na+ form.

Then, the product was purified by a RP-C18 column with (10%- 60% MeOH in water) to obtain 38 mg of compound **56** with a 65% yield. MS m/z 723.3 $[M]^-$.

Compound (**58**). Compound **56** (38mg, 0.0524 mmole) was dissolved in 5 mL 1/1 tBuOH/water. To it, 57 mg Pd/C (10%) was added and then a balloon of H2 was applied to the reaction for 2 hours. The mixture was filtered through PTFE syringe filter with 1/1 MeOH/water. The solvent was dried and redissolved in distilled water (5 mL). 57 mg of Pd(OH)2/C (20%) was added and H2 balloon was introduced to the reaction again. The reaction was left overnight at RT. Next day, it was filtered through PTFE syringe filter with water. The crude product was passed through a column of cation exchange resin Na+ form. Then, 20.8 mg of compound **58** was obtained with 79% yield after lyophilization. MS m/z 477.0 $[M]^-$.

Compound (**60**) (GlcNS-IdoA disaccharide substrate). Compound **58** (1.79mg, 0.0034 mmole) was dissolved in 0.5 mL anhydrous DMF and compound **51** (1.2 eq, 1.69 mg) was added to the mixture. The reaction was left for 2 days and then purified with HPLC to afford 0.4 mg compound **60** with a 14 % yield. MS m/z 841.6 $[M+Na]^+$.

3, 4, 6-Tri-*O*-acetyl-2-azido-2-deoxy-1-α-(2-naphthyl)-glucopyranoside (62). Compound **5** (1eq, 8g, 24 mmol), 2-naphthol (1.5 eq, 5.2 g) and 2,6-di-tert-butyl-4-methylpyridine (3 eq, 14.8 g) were dried for 3 hours under high vacuum (oil pump) and dissolved in anhydrous DCM (150 mL) under argon atmosphere. To the DCM solution, $BF_3 \cdot Et_2O$ (3 eq, 9.13 mL) was added dropwise with stirring at room temperature. After the reaction mixture had been stirred overnight

at room temperature, saturated aqueous NaHCO3 was added. The aqueous layer was extracted with DCM and the organic extracts were combined and washed with water, brine and dried over anhydrous Na_2SO_4. The solution was decanted and concentrated by rotary evaporation. Thin Layer Chromatography (TLC) with 3/1 hexane/EtOAc showed the separation between alpha and beta isomer with the R_f value of 0.32 and 0.25, respectively. The residue was purified by silica gel column chromatography (0-30% EtOAc in Hexane) to afford compound **6** (4.0 mg) in 36% yield as white solid (total alpha and beta isomer yield was 85% with a 36/49 alpha to beta ratio). Excessive silica gel was needed to sufficiently separate alpha and beta isomers. Alpha isomer was eluted first, and there was no baseline separation between the two isomers. Thus, NMR or HPLC-MS were recommended for the last few tubes of alpha isomer to confirm the purity.

MS *m/z* 480.4 $[M+Na]^+$.

^{1}H NMR (300 MHz, $CDCl_3$)(α isomer): δ 7.80 (m, 3H), 7.55 – 7.38 (m, 3H), 7.32 (m, 1H), 5.83 – 5.74 (m, 2H), 5.19 (t, *J* = 9.7 Hz, 1H), 4.33 (dd, *J* = 12.3, 4.7 Hz, 1H), 4.22 (dd, *J* = 10.3, 3.8 Hz, 1H), 4.09 (d, *J* = 9.1 Hz, 1H), 3.56 (dd, *J* = 10.6, 3.3 Hz, 1H), 2.16 (s, 3H), 2.07 (s, 3H), 2.02 (s, 3H).

1H NMR (300 MHz, CDCl3) (β isomer): δ 7.79 (m, 3H), 7.55 – 7.36 (m, 3H), 7.28 (m, 1H), 5.11 (m, 2H), 5.01 (d, *J* = 8.1 Hz, 1H), 4.31 (dd, *J* = 12.2, 5.6 Hz, 1H), 4.18 (m, 1H), 3.84 (m, 2H), 2.13 (s, 3H), 2.10 (s, 3H), 2.06 (s, 3H).

2-azido-2-deoxy-1-α-(2-naphthyl)-glucopyranoside (63). Compound **62** (1 eq, 4.0 g, 8.7 mmol) was dried under high vacuum for 3 hours and then dissolved in anhydrous MeOH under an argon atmosphere. To the solution, a catalytic amount of sodium methoxide was added. The reaction was allowed to stir at room temperature overnight under argon. Acetic acid was used to neutralize the reaction. Then, the reaction mixture was concentrated with rotary evaporation and

subjected to next step. MS *m/z* 354.4 $[M+Na]^+$.

2-amino-2-deoxy-1-α-(2-naphthyl)-glucopyranoside (64). The crude compound **63** was dissolved in THF (200 mL) and NaOH (0.1 M, 83 mL) was added to the solution. While the reaction was stirring at room temperature, trimethylphosphine in THF (1M, 41.5 mL) was added dropwise and the reaction was left stirring for 2 hours. The reaction was quenched by adding acetic acid to neutral pH and the solvent was removed with rotary evaporation. No further purification was needed, and the crude product was subjected to the next step. MS *m/z* 306.2 $[M+Na]^+$.

2-sulfamate-2-deoxy-1-α-(2-naphthyl)-glucopyranoside (65). The crude compound **64** was dissolved in 100mL water and sulfur trioxide pyridine complex (5 eq, 7.8g) was added to the solution in portions. Then, the pH of the reaction was adjusted by adding 4M aqueous NaOH to about 8.5-9. The color change could be a good indicator of the pH. The reaction started out as a light-yellow solution, and it turned into a dark red color when the pH was adjusted to around 9. The pH needs to be checked every 10 minutes until it was stable and then the reaction was left for 1 day until completion. Another 5 eq of sulfur trioxide pyridine could be added if the reaction did not reach completion after 1 day. Upon completion, the reaction was dried down with rotovap, and then dissolved in methanol (product will dissolve in MeOH but most of the salt will not); the mixture was then filtered, and the filtrate was dried down. The mixture was then dissolved in small amount of water (just enough to dissolve all the solid); then the solution was subjected to a short column (30g) of RP C18 to get rid of most of the ions in the product mixture. Elute the column first with pure water. Then elute the product with 10% MeOH in water. The

product was collected and pooled. The solvent was evaporated; then the solid was dissolved in MeOH and subjected to Q-Sepharose fast flow ion exchange column (strong anion exchanger) (HiLoad^{TM} 26/10 Q Sepharose High performance, Code No. 17-1066-01). Solvent A is pure MeOH; solvent B is 1 M ammonium formate in MeOH. The column was first eluted with 100% solvent A for 30 min to get rid of the residual starting material. Then, a gradient of 0-100% solvent B in 10 min was executed. Finally, the product was eluted with 100 % solvent B in 1 hours. Tubes that contained product were combined and concentrated with rotary evaporation at room temperature. It is crucial to not use heat to accelerate the drying process. Then the product was subjected to a desalt process with C18 sep-pak column. The product came out right after the ammonium formate salt with water as eluent. Once all the salt was gone, 10% MeOH in water was used to facilitate the elution of the product. Due to the low enzyme activity of sulfamidase, the final product must be pure. Target purity is less than 0.0027% mole fraction of compound **64** in compound **65**. To ensure the purity of final compound, it was purified through another short column of SP-Sepharose fast flow ion exchange resin (strong cation exchanger) if necessary. (most of the time, the anion exchange column is sufficient). The final substrate, compound **9**, was obtained as a white solid upon lyophilization. MS *m/z* 384.3$[M]^-$.

^{1}H NMR (300 MHz, MeOD) δ 7.78 (m, 3H), 7.62 (m, 1H), 7.46 – 7.32 (m, 3H), 5.97 (d, J = 3.4 Hz, 1H), 3.92 – 3.83 (m, 1H), 3.74 (m, 3H), 3.60 – 3.45 (m, 2H).

6. Discussions and conclusion

In this study, the first assay of sulfamidase in DBS for evaluation of MPS-IIIA is accomplished. All other previously reported sulfamidase assays made use of blood leukocytes or skin fibroblasts. The assay reported herein is appropriate for high-throughput NBS laboratories

and reference laboratories.

The novel substrate exhibits higher V_{max} and K_m in a DBS assay for sulfamidase, which agrees with Karpova's[18] finding. The extension of saccharide unit in the substrate for MPS III A lowers its K_m significantly but it does not necessarily correlate to a higher V_{max}. The reason behind this finding is unknown. With considerably lower cost of synthesizing the 2-naphthol substrate compared to the polysaccharide substrate, a higher substrate concentration in the actual assay is still affordable.

Since the new sulfamidase assay is carried out with LC-MS/MS, it will be possible to add it to our other LC-MS/MS assays for LSDs[11,44] to continue to expand the multiplex capabilities of this platform for NBS and diagnosis of lysosomal storage diseases. We plan to initiate pilot studies using the new sulfamidase assay in a NBS lab to explore the robustness of the newly developed MPS III A assay.

Biomarkers were also explored in this study. The NRE biomarker has shown its potential for a specific MPS III A biomarker despite all the drawbacks. As technology improves, the sensitive issue will be resolved and this NRE method may well be a reliable MPS III A diagnosis tool.

Chapter II. Development of Newborn Screening Method for Mucopolysaccharidosis III Type B in Dried Blood Spot using Tandem Mass Spectrometry

7. Introduction

Mucopolysaccharidosis III type B (MPS III B) is an autosomal recessive inherited disease with mild somatic and severe neurological manifestation.[5] It is caused by the defect in an enzyme called alpha-N-acetylglucosaminidase (NAGLU, EC 3.2.1.50). Due to the lack of function of NAGLU, MPS III B patients are not able to degrade a glycosaminoglycan named heparan sulfate. Infants who are affected by MPS III B usually will not live through the second decade of their lives.[36,45] There is no current treatment for MPS III B; however, intra-cerebrospinal fluid gene therapy and insulin growth fact II fusion protein intrathecal enzyme replacement therapy has shown promising early clinical results.[25,46] With future treatment in mind, MPS III B patients are still facing yet another problem: the diagnosis of this disorder. Because of the fast progression of MPS III B, the damage to the nervous system the patients sustained by the time the diagnosis was made (usually, when the symptom started to manifest) is already beyond repair. This necessitates an early diagnosis for MPS III B just as the case for MPS III A. The best chance for the patient to be caught soon after birth is to incorporate MPS III B into the Newborn Screening Project (NBS).

8. Alpha-N-acetylglucosaminidase and its functions

alpha-N-acetylglucosaminidase is an enzyme that catalyzes the hydrolysis of the terminal non-reducing end N-acetylglucosamine on a heparan sulfate molecule. Heparan sulfate is a polysaccharide that is consisted of repeating disaccharide unit (Figure 1). Glucosamine unit in

heparan sulfate has different substitutions on 2-, 3- and 6- position; uronic acid has a different substitution on 2- position as well. The variety of the substitution gives heparan sulfate its structural diversity, which is closely related to its biological function. Moreover, a series of enzymes are required to completely hydrolyze heparan sulfate because of this structure diversity. alpha-N-acetylglucosaminidase is one of the many enzymes that are responsible for this action. Since all of the enzymes act as an exoenzyme, the whole degradation of heparan sulfate will come to a stop when one enzyme is not functioning properly.[22]

In the event of alpha-N-acetylglucosaminidase malfunctioning or absence, the lysosome cannot cleave the N-acetylglucosamine residue from HS. The retention of the N-acetylglucosamine residue causes a complete halt of heparan sulfate degradation and storing of heparan sulfate inside the lysosome, which leads to a disease named mucopolysaccharidosis III type B (MPS III B).[47] The relevance between the storing of heparan sulfate and the pathology of MPS III B is still unknown but this does not undermine the significance of this disease as it is fatal and untreatable as of today.

With ongoing effort to develop a treatment for MPS III B,[25,46] a robust method for detecting this disease is also in demand. As mentioned above, the degradation of heparan sulfate requires collaboration between different enzymes. Any one of the enzymes' malfunction can cause the accumulation of heparan sulfate in cells and leads a nonspecific spectrum of symptoms. Thus, the diagnosis of MPS III B can be very difficult and the delay in diagnosis usually means the failure of a treatment if one is available. In order to specifically identify MPS III B from all other mucopolysaccharidosis (MPS), an enzymatic assay in DBS with tandem mass spectrometry (MS/MS) was proposed, but a viable substrate for sulfamidase must be found for this method to work.

9. Development of a suitable substrate for alpha N-acetylglucosaminidase

9.1. Alpha N-acetylglucosaminidase substrate with a mass spectrometry (MS) signal enhancer aglycone structure

9.1.1. Synthesis of the MPS III B substrate

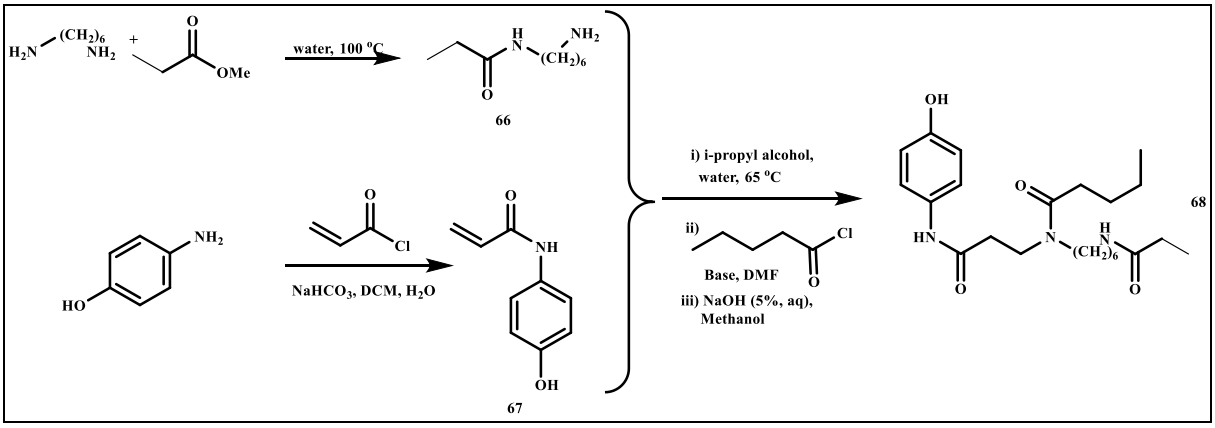


Figure 34. Synthesis of the MPS III B aglycone

The glycosyl donor for MPS III B substrate is the same as MPS III A substrate, compound **5**. Hexane-1,6-diamine and ethyl acetate were stirred in 100 °C water for 24 hours in order to yield compound **66**. Compound **67** was prepared by a nucleophilic acyl substitution between 4-aminophenol and acryloyl chloride. The precursor of the final aglycone was yielded by a Michael addition between **66** and 67; then, finally the aglycone, compound **68**, was achieved by adding the pentanoyl moiety to the secondary amine.[5] To yield compound **69**, boron trifluoride diethyl etherate was used to conjugate the sugar moiety and aglycone.[28] Compound **70** was obtained by reducing the azido group to the amine. Until this step, all the products were mixtures containing both alpha and beta glycosides. In order to separate the alpha and beta isomers of **71**, a trifluoroacetic anhydride was used to acetylate the amine, which then allowed the separation by

silica column chromatography. The final sulfamidase substrate, compound **73** was achieved after 2 steps: deacetylation and selective acetylation.[5]

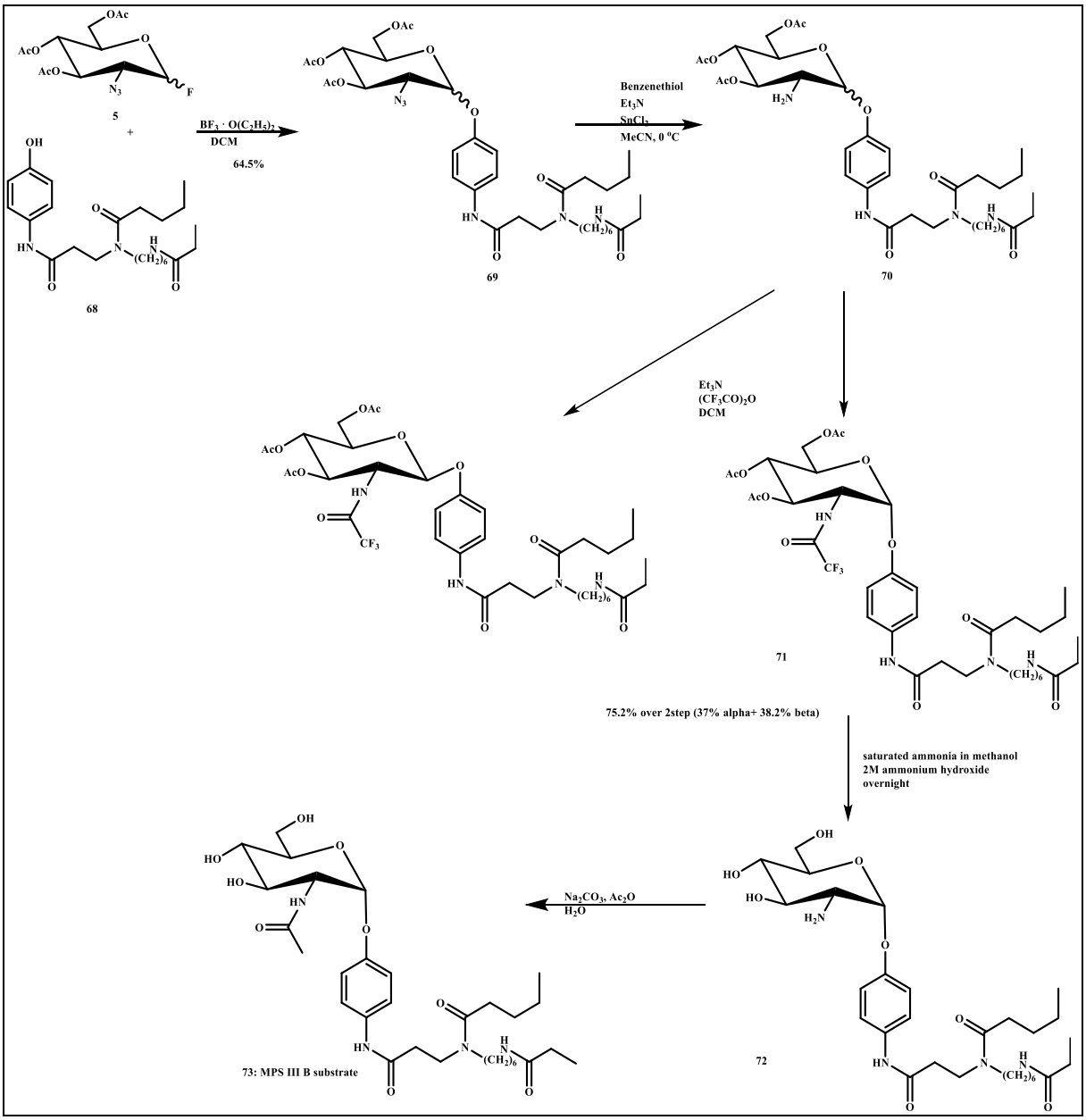


Figure 35. Synthesis of final MPS III B substrate

9.1.2. MPS III B assay with MPS III B substrate

With previous reports about NAGLU assay[5], the initial assay buffer condition was

established as follow: 50 mM sodium acetate, 7.5 mM barium (II) acetate, 5 mM cerium (III) acetate and pH 4.5. The assay used a 3 mm dried blood spot (DBS) punch (equivalent to 3.2 µL of blood) in a 96 deep well plate. To the DBS punch, 30µL of buffer containing 0.5 mM MPS III B substrate and 1 µM MPS I internal standard (used as a tentative IS) was added and put in the 37 °C orbital shaker for 16 hours at 250 rpm. The assay was then quenched by adding 120 µL of acetonitrile to each well, followed by 5 minutes of centrifugation at 3000 rpm to pellet the precipitate. 120 µL of the supernatant was then transferred to a shallow well plate and 120µL water was added to the plate. Then the plate was sealed with aluminum foil and subjected to LC/MS/MS directly. Mass spectrometry was performed on a Waters Xevo instrument using positive ion mode selected reaction monitoring (SRM) with tuned parameters that optimize the signal from the enzymatic product.

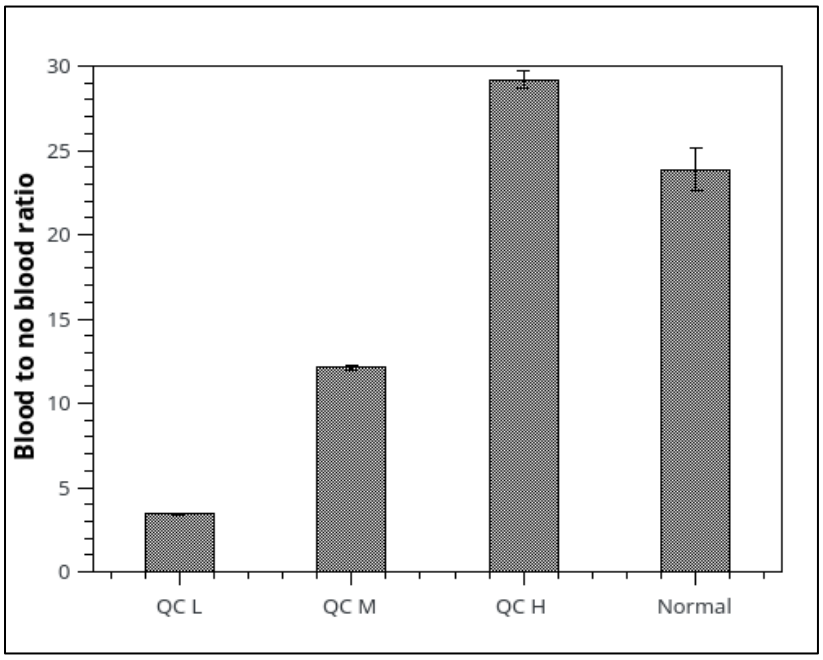


Figure 36. CDC QC DBS assay with MPS III B substrate at pH 4.5. QC L: quality control low; QC M: quality control medium; QC H: quality control high; Normal: normal blood.

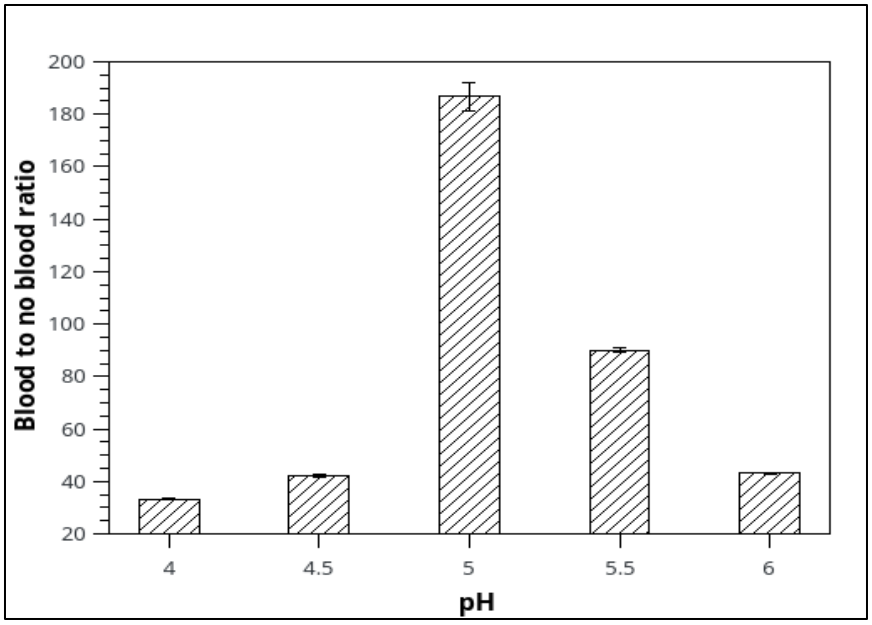


Figure 37. pH optimization of MPS III B DBS assay

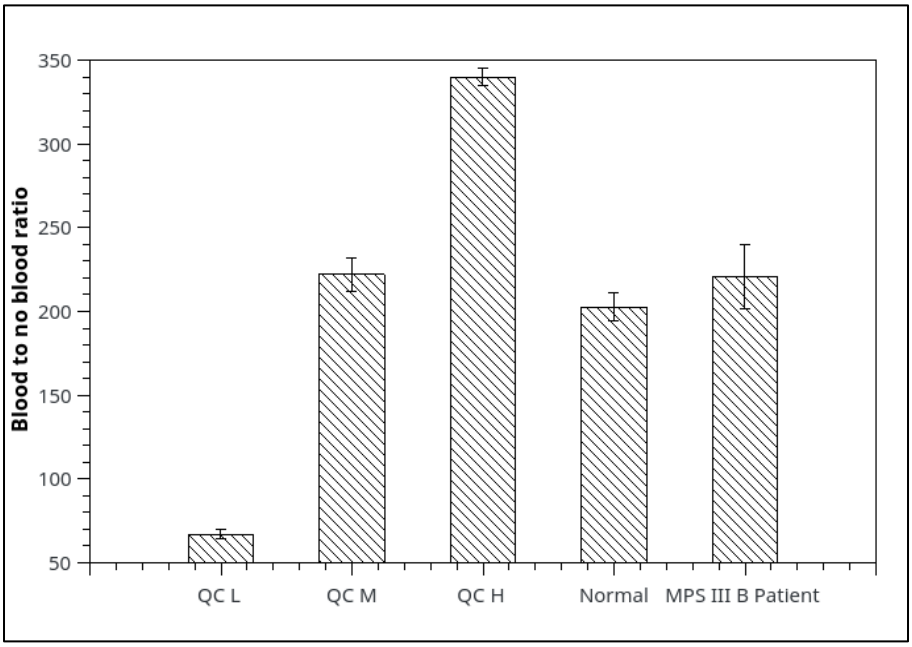


Figure 37. MPS III B patient assay. As it shows that the MPS III B patient has the same level of blood to no blood ratio as the normal DBS.

As shown in figure 36, a positive correlation between the content of normal leukocyte and the NAGLU activity and the normal DBS shown level of NAGLU activity between QC M

and QC H. This result was to be expected for a working assay for MPS III B. The optimal pH for MPS III B assay appeared to be at pH 5 as shown in Figure 37.

With the new pH for MPS III B, an experiment was conducted with MPS III B patient DBS to see the specificity of this assay. As shown in Figure 37, the MPS III B patient had a similar activity of NAGLU as a normal person. There are two probable causes for this finding: first, the substrate is not specific to NAGLU; secondly, there is impurities in the newly synthesized substrate, which is not a substrate to NAGLU but to other enzymes.

9.1.3. Inhibition of hexosaminidase in MPS III B assay

Hexosaminidase is a glycosidase that recognizes beta N-acetylhexose. In the synthetic steps for MPS III B substrate, trifluoroacetyl group was introduced to the free amine for a separation between the alpha and beta isomers at the anomeric position.

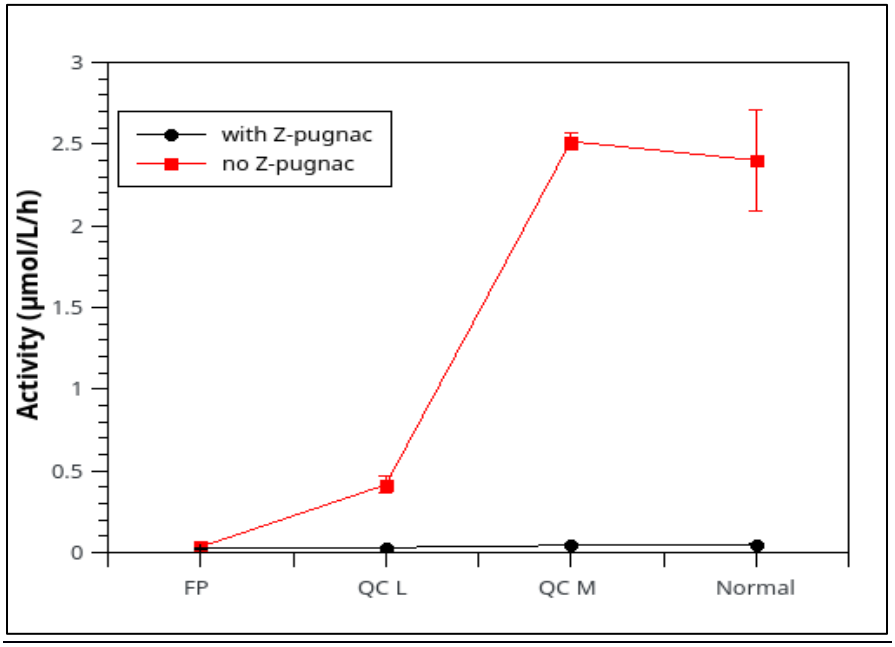


Figure 39. The inhibition of hexosaminidase by Z-pugnac. 200 µM Z-pugnac was used in the assay.

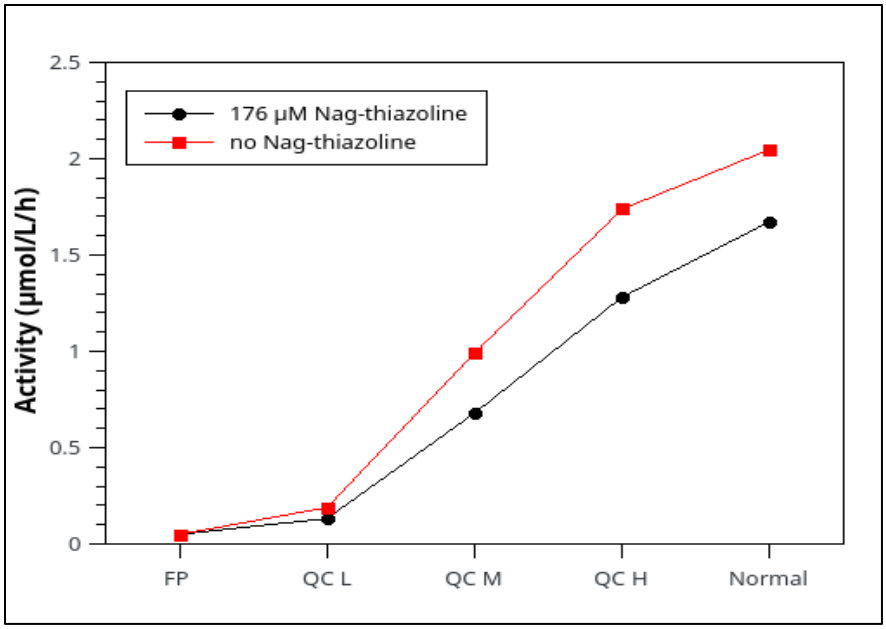


Figure 40. The inhibition of hexosaminidase by nag-thiazoline. 176 µM nag-thiazoline was used in the assay.

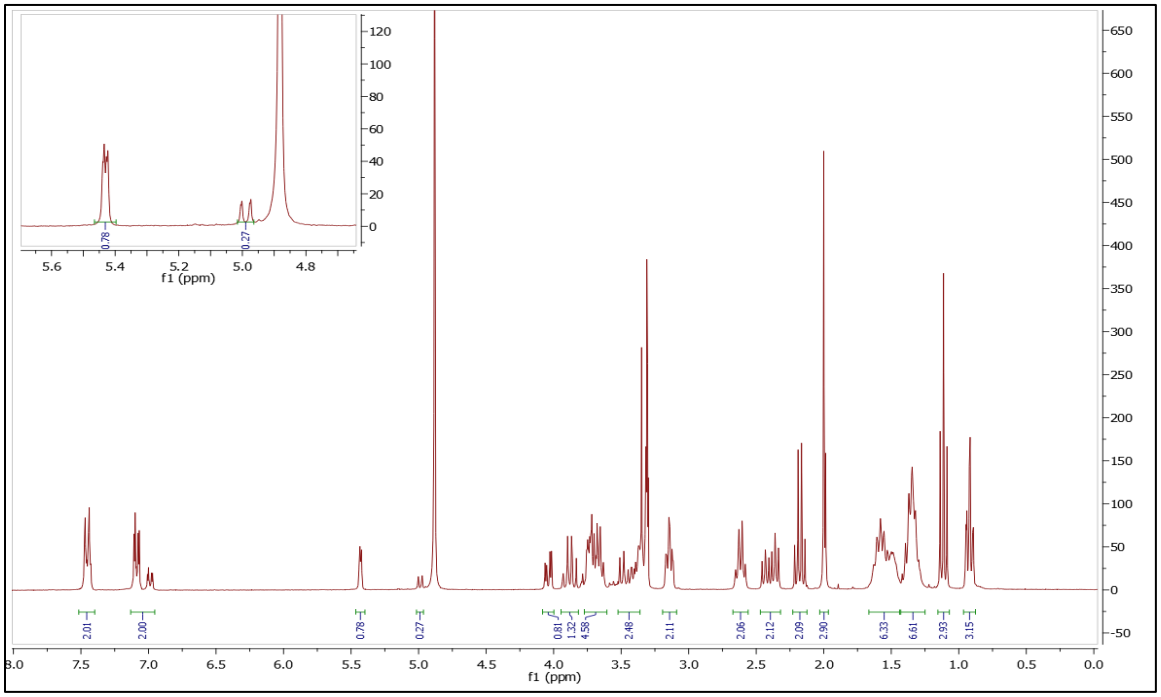


Figure 41. NMR spectrum for the first batch of MPS III B substrate. (In the upper left window: a zoomed-in vision of chemical shift 4.6-5.6. The first peak corresponding to alpha isomer anomeric proton as the J-coupling constant is 3.5; the second peak corresponding to beta isomer anomeric proton as the J-coupling constant is 8.5)

The importance of this step is shown here. When the separation of alpha and beta isomers

is not complete (figure 41), the residual beta isomers will become a substrate for hexosaminidase and generate the same enzymatic product as NAGLU. Fortunately, hexosaminidase can be inhibited by inhibitors.

Two of the inhibitors were used against hexosaminidase: Z-pugnac and nag-thiazoline. As shown in figure 39, the inhibitory effect of Z-pugnac was studied. Though it was a viable hexosaminidase inhibitor, Z-pugnac also inhibited NAGLU as shown in the black dot line. When incubated with 200 μM Z-pugnac, there was no activity of both NAGLU and hexosaminidase, which means Z-pugnac cannot be used as an inhibitor for hexosaminidase in MPS III B DBS assay. However, nag-thiazoline showed specificity for hexosaminidase. As shown in figure 40, when the assay was run with 176 μM nag-thiazoline, it still showed enzymatic product formation though it was lower than the assay without nag-thiazoline. Clearly, nag-thiazoline is the choice of inhibitor for hexosaminidase in MPS III B assay.

As mentioned above, hexosaminidase only affects the MPS III B assay when there is beta isoform of the MPS III B substrate presents. Theoretically, if the NAGLU substrate can be purified then there will be no need for the inhibitor.

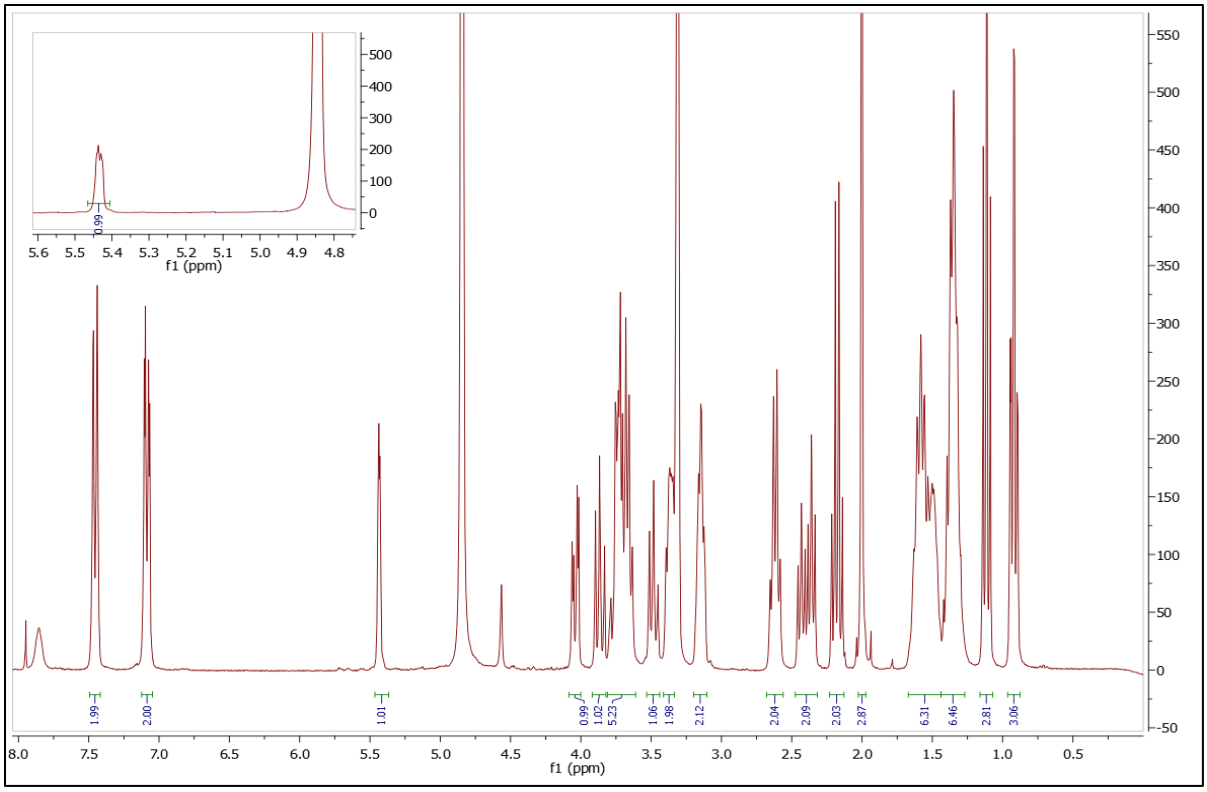
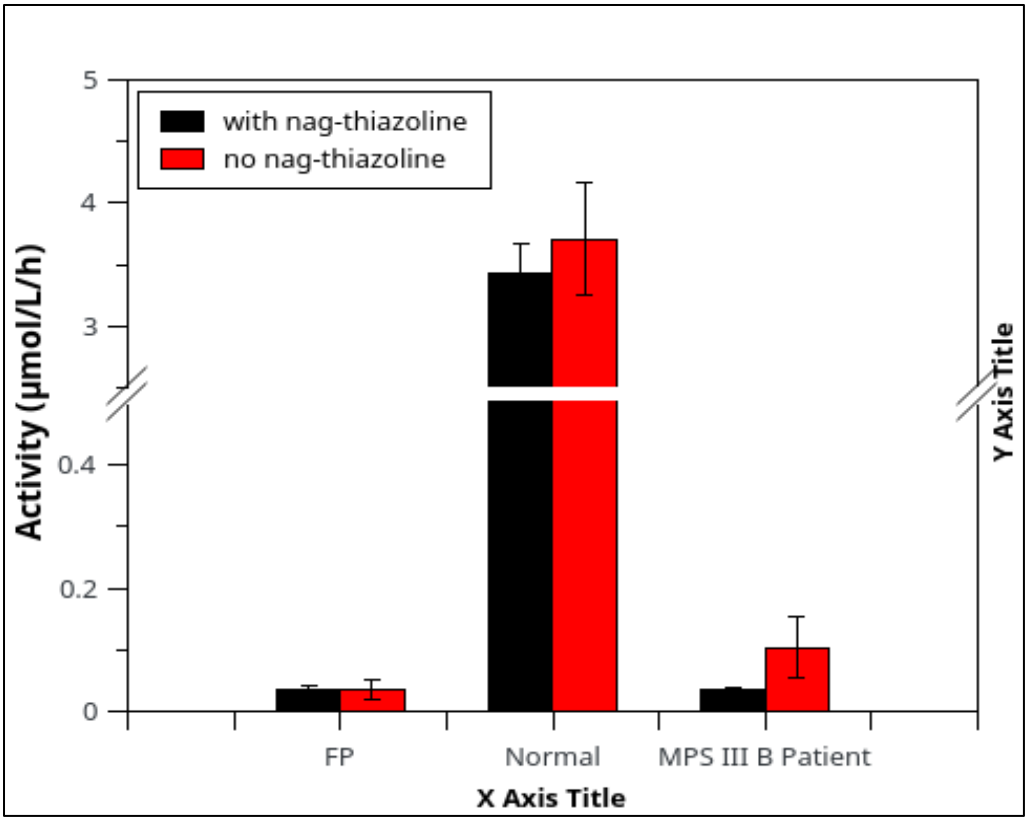


Figure 42. NMR spectrum for the purified batch of MPS III B substrate. (In the upper left window: a zoomed-in vision of chemical shift 4.6-5.6. The first peak corresponding to alpha isomer anomeric proton as the J-coupling constant is 3.5; there is no peak corresponding to beta isomer anomeric proton.)

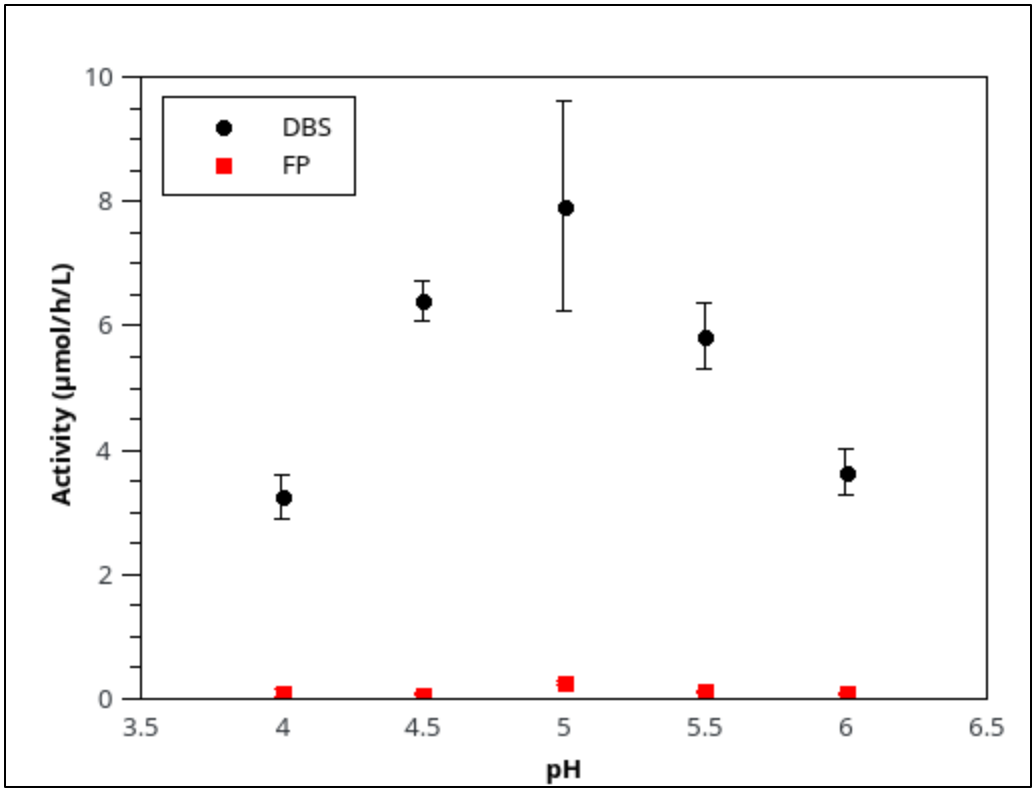
After further effort to purify the NAGLU substrate, a final batch with no visible beta isomers from the NMR was obtained (figure 42). Then, the purified substrate was subjected to a MPS III B patient assay. The result is shown in figure 43. Even though there was no visible beta isomer present in the substrate from a NMR spectrum, there is still product formation from hexosaminidase.

Since there is no way to make sure every batch of MPS III B substrate will have the same content of beta isomers of MPS III B substrate, it is necessary to have the inhibitor for hexosaminidase present in a MPS III B assay.



	activity(umol/h/L)		blood/no blood		SD		%CV	
Sample Name	w/o Nag	200 uM Nag	no nag	200uM nag	no nag	200uM nag	no nag	200uM nag
FP	0.0073	0.0065			0.0012	0.0011	16.13	16.85
MPS III B Patient	0.0642	0.0041	8.74	0.64	0.0027	0.0011	4.17	26.83
Normal	0.5460	0.5373	74.42	82.76	0.0573	0.0940	10.49	17.49

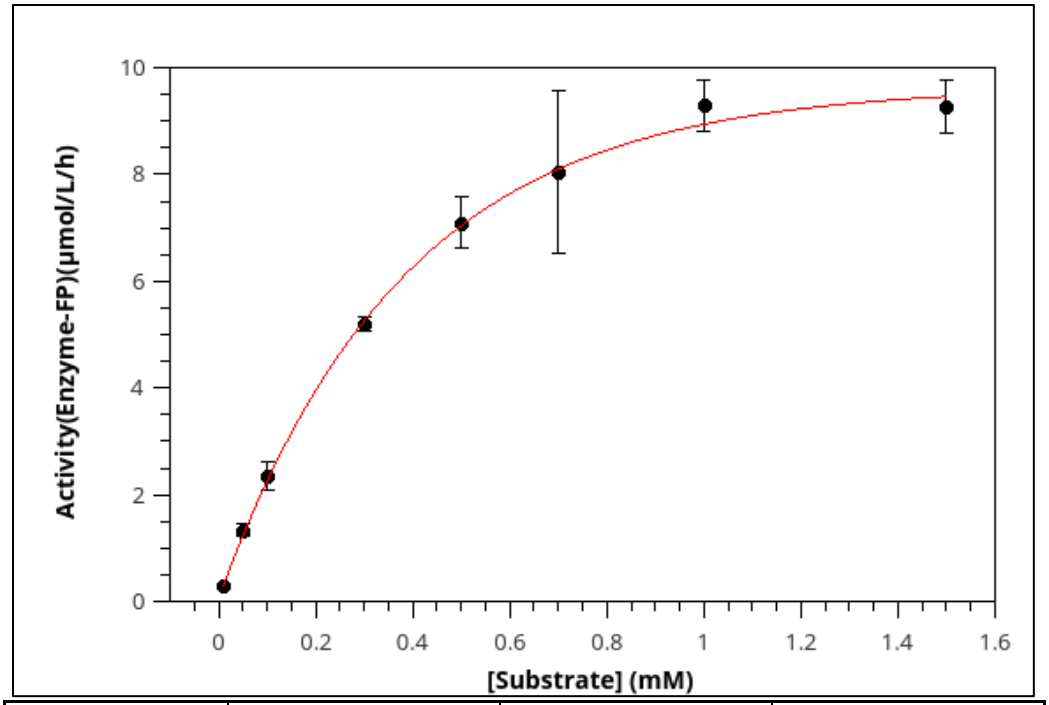
Figure 43. MPS III B patient assay with 200 uM nag-thiazoline. Even though there was no visible beta isomer present in the substrate from a NMR spectrum, there is still product formation from hexosaminidase.



	Activity (µmol/h/L)		SD		%CV	
pH	FP	DBS	FP	DBS	FP	DBS
4	0.102	3.246	0.065	0.349	63.52	10.76
4.5	0.075	6.391	0.021	0.314	27.74	4.92
5	0.258	7.920	0.022	1.689	8.37	21.33
5.5	0.119	5.823	0.007	0.530	6.13	9.11
6	0.091	3.649	0.013	0.375	13.87	10.28

Figure 38. pH optimization of MPS III B DBS assay

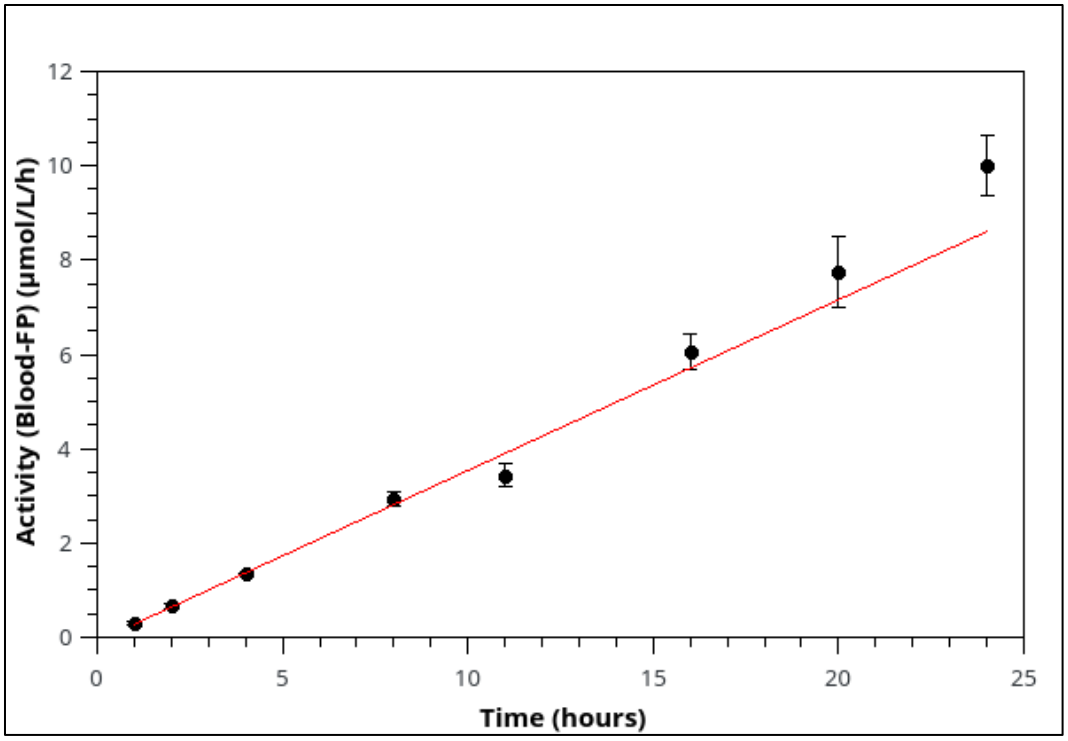
To finalize the MPS III B DBS assay condition, pH was optimized to 5.0 as shown in figure 44. As summarized in figure 45, NAGLU substrate concentration was plotted against NAGLU activity. The estimated K_m for NAGLU is 0.5 mM and V_{max} is 0.42 mM from a Lineweaver-Burk plot. Unlike the MPS III A assay, the activity of NAGLU is sufficient even with a value that's significantly lower than the K_m. To keep the assay in a consistent format, a substrate concentration of 0.5 mM was chosen for all future study. However, for practical purpose, this substrate concentration can be modified according to the cost and needs of the screening process.



	Activity (µmol/h/L)		SD		%CV	
[substrate] (mM)	FP	DBS	FP	DBS	FP	DBS
0.01	0.013	0.304	0.002	0.038	16	12
0.05	0.024	1.352	0.004	0.115	16	9
0.1	0.084	2.396	0.011	0.266	13	11
0.3	0.151	5.347	0.016	0.118	11	2
0.5	0.123	7.276	0.028	0.484	23	7
0.7	0.288	8.339	0.093	1.521	32	18
1	0.318	9.741	0.114	0.476	36	5
1.5	0.437	9.822	0.061	0.496	14	5

Figure 395. substrate concentration curve for MPS III B DBS assay. Large CV is to be expected for FP sample.

Further investigation regarding the incubation times was also conducted. As shown in figure 46, there was a linear relationship between the incubation time and amount of NAGLU enzymatic product formed up to 24 hours. For practical purpose, 16 hours was chosen as the incubation time for MPS III B assay.



Time (h)	Product formed (nmole)	SD	CV
1	0.015	0.002	10.25
2	0.034	0.003	9.00
4	0.069	0.001	1.57
8	0.150	0.008	5.42
11	0.176	0.013	7.20
16	0.310	0.019	6.11
20	0.397	0.039	9.75
24	0.513	0.032	6.34

Figure 40. Time course for MPS III B DBS assay

One big advantage of using MS/MS for screening is the ability of multiplexing with other assays. With help from Yang Liu[11], MPS III B was successfully included into a 6-plex assay with 5 other enzymes: tripeptidyl peptidase 1 (TPP1); α-N-acetylglucosaminidase (NAGLU); lysosomal β-glucuronidase (GUSB); iduronate-2-sulfatase (I2S); N-acetylgalactosamine-6-sulfatase (GALNS); and arylsulfatase B (ARSB). The running time for this 6-plex assay is exactly the same as the single assay with MPS III B. As shown in figure 47, all enzymatic products were

separated from their corresponding substrate to eliminate any insource break down of the substrate. “The assays were performed in 96-well plates in 50 mM ammonium acetate buffer (pH 5.0) with an assay cocktail consisting of substrates at the following concentrations: TPP1 (0.2 mM), I2S (1.0 mM), NAGLU (0.5 mM), GALNS (1.0 mM), ARSB (1.0 mM), and GUSB (0.5 mM)” as stated by Liu.[11] The assay cocktail also contained corresponding internal standards for all enzymes: 15 μM TPP1 IS, 10 μM I2S IS, 10 μM NAGLU IS, 7.5 μM GALNS IS, 10 μM ARSB IS, and 10 μM GUSB IS with additional 100 μM NAG-Thiazoline for inhibition of hexosaminidase.[48,49] One 3 mm DBS punch was placed in each well with 30 μL of the cocktail mentioned above. The plate was then covered with plate sealer and left in a 37 °C orbital shaker (250 rpm) for 16 hours. The incubation was quenched with 100 μL 50/50 MeOH/EA. All of the products and ISs were tested before and the majority of them preferred to go into the EA layer while all the substrates and salts preferred to stay with the aqueous layer. The EA layer was then dried with N_2 jet. The residue was then reconstituted with elution solvent for the UPLC, 55/45/0.1 water/acetonitrile/formic acid, and injected into the UPLC-MS/MS. The elution was done in an isocratic fashion with 55/45/0.1 water/acetonitrile/formic acid. All the analysts were then monitored in MS using MRM mode. Comparing this multiplex assay procedure with the one for MPS III A and B, the multiplex assay can save tremendous time and cost for a large-scale screening project.

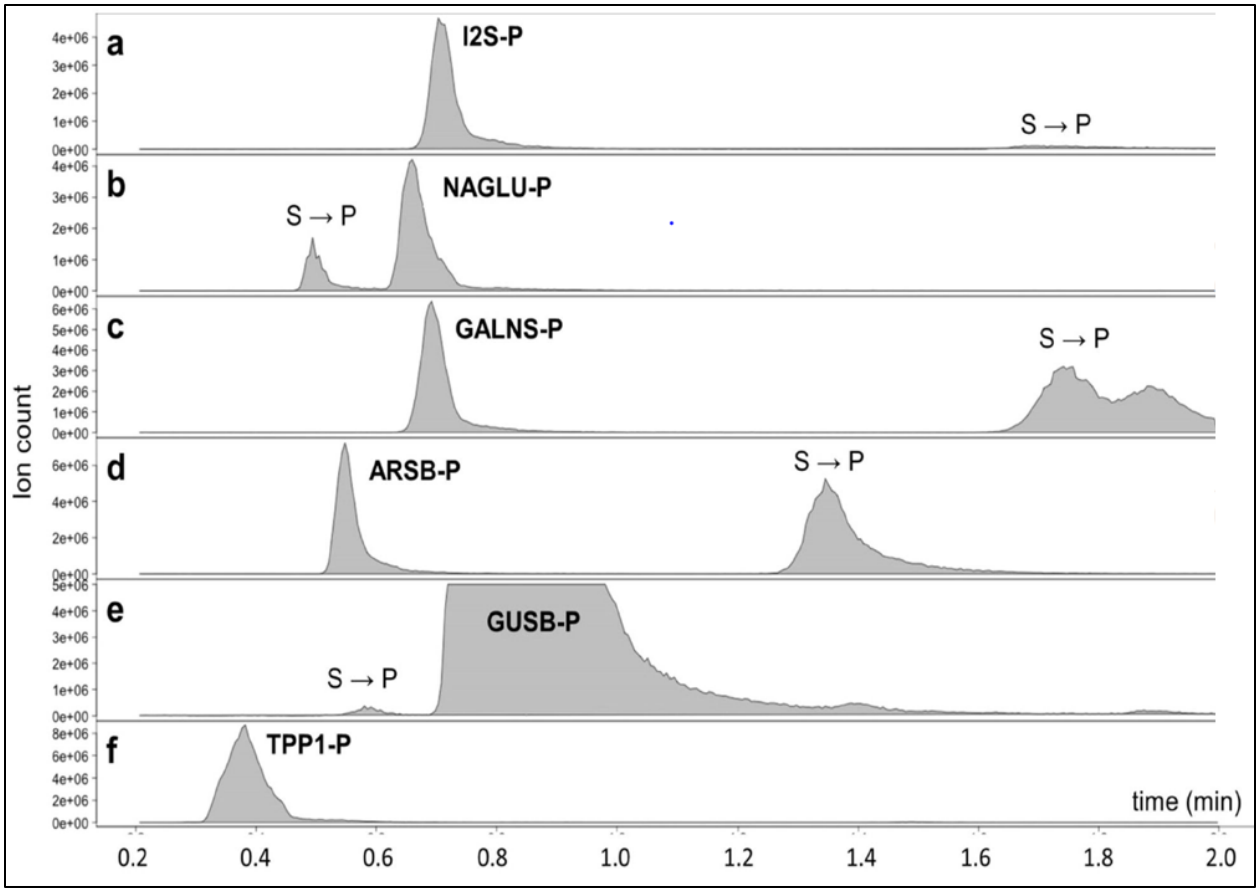


Figure 47. Multiplex of 6 enzymatic assay. (TPP1: tripeptidyl peptidase 1; NAGLU: α-N-acetylglucosaminidase; GUSB: lysosomal β-glucuronidase; I2S: iduronate-2-sulfatase; GALNS: N-acetylgalactosamine-6-sulfatase; and ARSB: arylsulfatase B.) (This graph is the courtesy of Yang Liu, detail see reference 11)

10. Synthesis of compound involved in MPS III B assay

N-(6-Amino-hexyl)-propionamide (66). To methyl propionate (11.8 mL, 122.4 mmol), hexane-1,6-diamine (14.2 g, 122.4 mmol) and water (6.2 mL) were added and the mixture was heated to 100°C for 24 hours under constant stirring. The reaction mixture was cooled to room temperature and directly loaded on to a short silica column. Upon elution with 10 to 20% of methanol (with 10% NH4OH) in DCM the desired mono-propionated product **66** was obtained (10.5 g, 60.9 mmol) in 49.7% yield.

^{1}H NMR (300 MHz, MeOD) δ 3.16 (t, *J* = 6.9 Hz, 2H), 2.63 (t, *J* = 7.0 Hz, 2H), 2.18 (q, *J* = 7.6 Hz, 2H), 1.58 – 1.26 (m, 8H), 1.12 (t, *J* = 7.6 Hz, 3H).

Pentanoic acid [2-(4-hydroxy-phenylcarbamoyl)-ethyl]-(6-propionylamino-hexyl)-amide (68). 4-Acrylamido-phenol (9.46 g, 58 mmol) and mono-propionyl-1,6-hexanediamine **66** (10.5 g, 60.9 mmol) were dissolved in a solution of isopropanol (450 mL) and water (50 mL) and heated in an oil bath at 65oC for 48 hrs. The reaction mixture was concentrated by rotary evaporation to afford the Michael addition product, which was used for the next step without further purification. To the residue from the above step was added CH2Cl2 (150 mL), DMF (15 mL) and 150 mL of saturated sodium bicarbonate in water. Pentanoyl chloride (7.96 mL, 67.1 mmol) was added dropwise at room temperature with stirring, and the mixture was stirred for an additional 6 h at room temperature. The organic layer was separated, and the water layer was extracted twice with 150 mL portions of 5% MeOH in CH2Cl2. The organic layers were combined and concentrated by rotary evaporation. The residue was purified by silica gel column chromatography (1-15% MeOH in CH2Cl2) to afford MPS –IIIB aglycone 6**8** (18.6 g, 44.3 mmol) in 76.4 % yield.

^{1}H NMR (300 MHz, MeOD) δ 7.31 (d, J = 8.9 Hz, 2H), 6.73(m, 2H), 3.76 – 3.61 (m, 2H), 3.36(m, 2H), 3.19 – 3.10 (m, 2H), 2.64 – 2.54 (m, 2H), 2.47 – 2.32(m, 2H), 2.22 – 2.12 (m, 2H), 1.68 – 1.23 (m, 12H), 1.11 (t, J = 7.6 Hz, 3H), 0.93 (t, J = 7.3 Hz, 3H). MS m/z 420.6 (M+H+).

Acetic acid 3-acetoxy-2-acetoxymethyl-5-azido-6-(4-{3-[pentanoyl-(6-propionylamino-hexyl)-amino]-propionylamino}-phenoxy)-tetrahydro-pyran-4-yl ester (69). MPS-IIIB agly-

cone **68** (5.67 g, 13.5 mmol, 1.1eq), 3, 4, 6-Tri-*O*-acetyl-2-azido-2-deoxy-1-fluoro- α/β -D-glucopyranoside **5** (4.1 g, 12.3 mmol, 1 eq) and 2,6-di-*tert*-butyl-4-methylpyridine (5.05 g, 24.6 mmol, 2 eq) were dried for 1 hr under high vacuum (oil pump) and dissolved in dry CH2Cl2 (615 mL, 0.02 M). BF3.Et2O (12.4 mL, 98.4 mmol, 8 eq) was added dropwise with stirring at room temperature under a nitrogen atmosphere. After the reaction mixture had been stirred for 2.5 h at room temperature, 350 mL of saturated aqueous NaHCO3 was added. The aqueous layer was extracted with CH2Cl2 and the organic extracts were combined and washed with water, brine and dried over anhydrous Na2SO4. The solution was filtered and concentrated by rotary evaporation. The residue was purified by silica gel column chromatography (CH2Cl2, then 1-10% MeOH in CH2Cl2) to afford product **69** (3.5 g, 4.77 mmol) in 39% yield. MS *m/z* 733.5 $[M+H]^+$.

Acetic acid 4-acetoxy-2-acetoxymethyl-5-amino-6-(4-{3-[pentanoyl-(6-propionylamino-hexyl)-amino]-propionylamino}-phenoxy)-tetrahydro-pyran-3-yl ester (70). Compound **69** (3.5 g, 4.77 mmol, 1 eq) was dissolved in dry acetonitrile (24 mL) under nitrogen atmosphere and was cooled in an ice bath. To this solution was added an ice-cold solution of tin(II) chloride (181 mg, 0.95 mmol, 0.20 eq), triethylamine (2 mL, 14.3 mmol, 3eq) and thiophenol (1.95 mL, 19 mmol, 4 eq) in 15 mL of dry acetonitrile. The solution was stirred at 0 °C for 2 hours and concentrated by rotary evaporation, diluted with dichloromethane then washed with cold 1N NaOH, water and brine. The organic layer was separated and concentrated by rotary evaporation, and the crude product was used for the next step without further purification.

Acetic acid 4-acetoxy-2-acetoxymethyl-6-(4-{3-[pentanoyl-(6-propionylamino-hexyl)-amino]-propionylamino}-phenoxy)-5-(2,2,2-trifluoro-acetylamino)-tetrahydro-pyran-3-yl ester (71). The crude product **70** was dried under high vacuum and dissolved in dry of dichloromethane (20 mL) and pyridine (3.84 mL, 47.7 mmol, 10 eq) was first added, followed by dropwise addition of trifluoroacetic anhydride (1 mL, 7.15 mmol, 1.5 eq). the resulted mixture was stirred for 4h at room temperature under nitrogen atmosphere. The solvent was removed by rotary evaporation, and the residue was purified using flash chromatography with 1-5% MeOH in DCM to give product **71** (α:β:1:1) (2.5 g, 3.1 mmol) in 65% yield.

^{1}H NMR (300 MHz, CDCl3) (α isomer): δ 7.54 (d, *J* = 9.0 Hz, 2H), 7.02 (d, *J* = 9.0 Hz, 2H), 5.57 (d, *J* = 3.5 Hz, 1H), 5.49(t, *J* = 9.6 Hz, 1H), 5.22 (t, *J* = 9.7 Hz, 1H), 4.52 – 4.41 (m, 1H), 4.26 (dd, *J* = 12.1, 4.4 Hz, 1H), 4.16 – 4.02 (m, 2H), 3.68 (t, J = 6.4 Hz, 3H), 3.32 – 3.19 (m, 4H), 2.66 (t, *J* = 6.4 Hz, 2H), 2.34 – 2.27 (m, 2H), 2.24 – 2.14 (m, 2H), 2.10 – 2.01 (m, 9H), 1.68 – 1.28 (m, 12H), 1.15 (t, *J* = 7.6 Hz, 3H), 0.90 (t, *J* = 7.3 Hz, 3H). MS *m/z* 825.6 $[M+Na]^+$.

Compound (72). compound **71** (281 mg, 0.35 mmol) was dissolved in 16 mL 7 N ammonia in methanol and 6 mL of 2 M ammonium water was added to the reaction. Reaction was then sealed and left at room temperature overnight. Next day, the reaction was dried and carried out the next step without further purification. MS *m/z* 581.3 $[M+H]^+$.

Compound (73). compound **72** (203 mg, 0.35 mmol) was dissolved in 150 mL water. To it, sodium carbonate (0.37 mg, 10 eq) and acetic anhydride (5 eq, 0.165 mL) were added. Reaction was left stirring for 4 hours at RT. The reaction was dried and purified by HPLC with C18 column to give product **73** (195.7 mg, 89.8%). MS *m/z* 645.7 $[M+Na]^+$.

^{1}H NMR (300 MHz, MeOD) δ 7.49 – 7.42 (m, 2H), 7.12 – 7.05 (m, 2H), 5.43 (d, *J* = 2.0 Hz, 1H), 4.04 (dd, *J* = 10.7, 3.5 Hz, 1H), 3.86 (dd, *J* = 10.7, 8.7 Hz, 1H), 3.81 – 3.61 (m, 5H), 3.48 (t, *J* = 9.1 Hz, 1H), 3.41 – 3.33 (m, 2H), 3.14 (dd, *J* = 6.6, 4.8 Hz, 2H), 2.62 (q, *J* = 7.0 Hz, 2H), 2.39 (dt, *J* = 21.3, 7.5 Hz, 2H), 2.18 (q, *J* = 7.6 Hz, 2H), 2.00 (s, 3H), 1.67 – 1.44 (m, 6H), 1.44 – 1.27 (m, 6H), 1.11 (t, *J* = 7.6 Hz, 3H), 0.92 (td, *J* = 7.2, 1.7 Hz, 3H).

11. Conclusions and discussions

In this study, the alpha-N-acetylglucosaminidase assay in DBS for evaluation of MPS-III B is redesigned. The assay reported herein is appropriate for high-throughput NBS laboratories and reference laboratories. A new aglycone structure was incorporated into MPS III B substrate for a higher sensitivity in MS/MS.

A higher signal for product is important in a screening process because the higher analytical range will give experts a better understanding of the prognosis of this disease. It enables the possibility of establishment early onset, late onset, and carriers of this disease. Healthcare professions can make medical decision accordingly regarding the timeline for treatment.

The multiplexity of MPS III B assay was also explored with other enzyme assays. The results in Liu and Yi's paper[11] clearly shows that the 6-plex assay works since all the enzymatic products can be separated from the corresponding substrates to eliminate the insource breakdown. Moreover, it also shows the ability of each enzyme to distinguish between the patients and normal persons.

References

1. Boustany, R. M. N. Lysosomal storage diseases - The horizon expands. *Nat. Rev. Neurol.* **9,** 583–598 (2013).
2. Desnick, R. J. & Schuchman, E. H. *Enzyme Replacement Therapy for Lysosomal Diseases: Lessons from 20 Years of Experience and Remaining Challenges*. *Annual Review of Genomics and Human Genetics* **13,** (2012).
3. Kirkegaard, T. Emerging therapies and therapeutic concepts for lysosomal storage diseases. *Expert Opin. Orphan Drugs* **1,** 385–404 (2013).
4. Burrow, T. A., Hopkin, R. J., Leslie, N. D., Tinkle, B. T. & Grabowski, G. A. Enzyme reconstitution/replacement therapy for lysosomal storage diseases. *Curr. Opin. Pediatr.* **19,** 628–635 (2007).
5. Wolfe, B. J. *et al.* New Substrates and Enzyme Assays for the Detection of Mucopolysaccharidosis III (Sanfilippo Syndrome) Types A, B, C, and D by Tandem Mass Spectrometry. *Bioconjug. Chem.* **23,** 557–564 (2012).
6. Zhang, F., Zhang, Z. & Linhardt, R. J. in *Handbook of Glycomics* 59–80 (Elsevier, 2010). doi:10.1016/B978-0-12-373600-0.00003-2
7. Bodamer, O. A., Giugliani, R. & Wood, T. The laboratory diagnosis of mucopolysaccharidosis III (Sanfilippo syndrome): A changing landscape. *Mol. Genet. Metab.* **113,** 34–41 (2014).
8. Almannai, M., Marom, R. & Sutton, V. R. Newborn screening: a review of history, recent advancements, and future perspectives in the era of next generation sequencing. doi:10.1097/MOP.0000000000000414
9. Mitchell, J. J., Trakadis, Y. J. & Scriver, C. R. Phenylalanine hydroxylase deficiency.

Genet. Med. **13,** 697–707 (2011).

10. Gelb, M. H., Scott, C. R. & Turecek, F. Newborn screening for lysosomal storage diseases. *Clin. Chem.* **61,** 335–46 (2015).
11. Liu, Y. *et al.* Multiplex Tandem Mass Spectrometry Enzymatic Activity Assay for Newborn Screening of the Mucopolysaccharidoses and Type 2 Neuronal Ceroid Lipofuscinosis. *Clin. Chem.* **63,** 1118–1126 (2017).
12. Chennamaneni, N. K. *et al.* Improved reagents for newborn screening of mucopolysaccharidosis types I, II, and VI by tandem mass spectrometry. *Anal. Chem.* **86,** 4508–14 (2014).
13. Muschol, N. *et al.* Transport, enzymatic activity, and stability of mutant sulfamidase (SGSH) identified in patients with mucopolysaccharidosis type III A. *Hum. Mutat.* **23,** 559–566 (2004).
14. Haurigot, V. *et al.* Whole body correction of mucopolysaccharidosis IIIA by intracerebrospinal fluid gene therapy. *J. Clin. Invest.* **123,** 3254–3271 (2013).
15. Jones, S. A. *et al.* A phase 1/2 study of intrathecal heparan-N-sulfatase in patients with mucopolysaccharidosis IIIA. *Mol. Genet. Metab.* **118,** 198–205 (2016).
16. Hopwood, J. J. & Elliott, H. Radiolabelled oligosaccharides as substrates for the estimation of sulfamidase and the detection of the Sanfilippo Type A syndrome. *Clin. Chim. Acta* **112,** 55–66 (1981).
17. Mandal, S. S. *et al.* Chemical synthesis of the tumor-associated globo H antigen. *RSC Adv.* **5,** 23311–23319 (2015).
18. Karpova, E. A., Voznyi, Y. V., Hoogeveen, A. T. & Winchester, B.van Diggelen, O. P. A fluorimetric enzyme assay for the diagnosis of Sanfilippo A disease (MPS III A). *Journal*

of inherited metabolic disease **19,** 278–285 (1996).

19. Levy, H. L. Newborn screening by tandem mass spectrometry: a new era. *Clin. Chem.* **44,** 2401–2 (1998).
20. Li, Y. *et al.* Direct Multiplex Assay of Lysosomal Enzymes in Dried Blood Spots for Newborn Screening. *Clin. Chem.* **50,** 1785–1796 (2004).
21. Potier, A. *et al.* Multiplex MS/MS method to measure MPS II, MPS IIIB, MPS IVA, MPS VI enzyme activities in dried blood spots. *Mol. Genet. Metab.* **117,** S97 (2016).
22. Gallagher, J. T. & Walker, A. Molecular distinctions between heparan sulphate and heparin. Analysis of sulphation patterns indicates that heparan sulphate and heparin are separate families of N-sulphated polysaccharides. *Biochem. J.* **230,** 665–74 (1985).
23. Ghosh, A. *et al.* Recommendations on clinical trial design for treatment of Mucopolysaccharidosis Type III. *Orphanet J. Rare Dis.* **12,** 117 (2017).
24. Mason, K., Meikle, P. J., Hopwood, J. & Fuller, M. Characterization of sulphated oligosaccharides in mucopolysaccharidosis type IIIA by electrospray ionization tandem mass spectrometry. *Ann. Chem.* **in press,** 4534–4542 (2006).
25. Beck, M. Treatment strategies for lysosomal storage disorders. *Dev. Med. Child Neurol.* **60,** 13–18 (2018).
26. Alper, P. B., Hung, S.-C. & Wong, C.-H. Metal catalyzed diazo transfer for the synthesis of azides from amines. *Tetrahedron Lett.* **37,** 6029–6032 (1996).
27. Dasgupta, F. & Masada, R. I. Synthesis of 7-O-(2-deoxy-2-sulfamido-alpha-D-glucopyranosyl)-4-methylcoumarin sodium salt: a fluorogenic substrate for sulfamidase. *Carbohydr. Res.* **337,** 1055–1058 (2002).
28. Chennamaneni, N. K. *et al.* Improved reagents for newborn screening of

mucopolysaccharidosis types I, II, and VI by tandem mass spectrometry. *Anal. Chem.* **86,** 4508–4514 (2014).

29. De Jesus, V. R. *et al.* Development and Evaluation of Quality Control Dried Blood Spot Materials in Newborn Screening for Lysosomal Storage Disorders. *Clin. Chem.* **55,** 158–164 (2008).

30. Freeman, C. & Hopwood, J. J. Human liver sulphamate sulphohydrolase structure on catalytic properties. **92,** 83–92 (1986).

31. Kuszmann, J., Medgyes, G. & Boros, S. Synthesis of 2,5-anhydro-(beta-d-glucopyranosyluronate)- and (alpha-l-idopyranosyluronate)-d-mannitol hexa-O-sulfonate hepta sodium salt. *Carbohydr. Res.* **339,** 1569–79 (2004).

32. Barroca, N. & Jacquinet, J.-C. Syntheses of β-d-GalpNAc4SO3-(1→4)-l-IdopA2SO3, a disaccharide fragment of dermatan sulfate, and of its methyl α-l-glycoside derivative. *Carbohydr. Res.* **329,** 667–679 (2000).

33. Arungundram, S. *et al.* Modular synthesis of heparan sulfate oligosaccharides for structure-activity relationship studies. *J. Am. Chem. Soc.* **131,** 17394–405 (2009).

34. Hopwood, J. J. & Elliott, H. Diagnosis of Sanfilippo type A syndrome by estimation of sulfamidase activity using a radiolabelled tetrasaccharide substrate. *Clin. Chim. Acta* **123,** 241–250 (1982).

35. Bielicki, J., Hopwood, J. J., Melville, E. L. & Anson, D. S. Recombinant human sulphamidase: expression, amplification, purification and characterization. *Biochem. J.* **329 (Pt 1,** 145–50 (1998).

36. de Ruijter, J. *et al.* Heparan sulfate and dermatan sulfate derived disaccharides are sensitive markers for newborn screening for mucopolysaccharidoses types I, II and III.

Mol. Genet. Metab. **107,** 705–10 (2012).

37. Kubaski, F. *et al.* Glycosaminoglycan levels in dried blood spots of patients with mucopolysaccharidoses and mucolipidoses. *Mol. Genet. Metab.* **120,** 247–254 (2017).

38. Rabenstein, D. L. Heparin and heparan sulfate: structure and function. *Nat. Prod. Rep.* **19,** 312–331 (2002).

39. Bultel, L., Landoni, M., Grand, E., Couto, A. S. & Kovensky, J. UV-MALDI-TOF Mass Spectrometry Analysis of Heparin Oligosaccharides Obtained by Nitrous Acid Controlled Degradation and High Performance Anion Exchange Chromatography. *J. Am. Soc. Mass Spectrom.* **21,** 178–190 (2010).

40. Wu, Z. L. & Lech, M. Modification degrees at specific sites on heparan sulphate: an approach to measure chemical modifications on biological molecules with stable isotope labelling. *Biochem. J.* **389,** 383–8 (2005).

41. Lawrence, R. *et al.* Evolutionary differences in glycosaminoglycan fine structure detected by quantitative glycan reductive isotope labeling. *J. Biol. Chem.* **283,** 33674–84 (2008).

42. Ruhaak, L. R., Steenvoorden, E., Koeleman, C. A. M., Deelder, A. M. & Wuhrer, M. 2-Picoline-borane: A non-toxic reducing agent for oligosaccharide labeling by reductive amination. *Proteomics* **10,** 2330–2336 (2010).

43. Shinjo, S. K. *et al.* Heparin and heparan sulfate disaccharides bind to the exchanger inhibitor peptide region of Na+/Ca2+ exchanger and reduce the cytosolic calcium of smooth muscle cell lines. Requirement of C4-C5 unsaturation and 1→4 glycosidic linkage for activity. *J. Biol. Chem.* **277,** 48227–48233 (2002).

44. Spacil, Z. *et al.* High-throughput assay of 9 lysosomal enzymes for newborn screening. *Clinical chemistry* **59,** 502–11 (2013).

45. Ficko-Blean, E., Stubbs, K. a, Nemirovsky, O., Vocadlo, D. J. & Boraston, A. B. Structural and mechanistic insight into the basis of mucopolysaccharidosis IIIB. *Proc. Natl. Acad. Sci. U. S. A.* **105,** 6560–6565 (2008).

46. Kan, S. *et al.* Insulin-like growth factor II peptide fusion enables uptake and lysosomal delivery of α-N-acetylglucosaminidase to mucopolysaccharidosis type IIIB fibroblasts. *Biochem. J.* **458,** 281–9 (2014).

47. Yogalingam, G. & Hopwood, J. J. Molecular genetics of mucopolysaccharidosis type IIIA and IIIB: Diagnostic, clinical, and biological implications. *Hum. Mutat.* **18,** 264–281 (2001).

48. Bayleran, J., Hechtman, P. & Saray, W. Synthesis of 4-methylumbelliferyl-β-d-N-acetylglucos-amine-6-sulfate and its use in classification of GM2 gangliosidosis genotypes. *Clin. Chim. Acta* **143,** 73–89 (1984).

49. Eun Ju Kim, †, Melissa Perreira, ‡, Craig J. Thomas, ‡ and & John A. Hanover*, †. An O-GlcNAcase-Specific Inhibitor and Substrate Engineered by the Extension of the N-Acetyl Moiety. (2006). doi:10.1021/JA0582915