Engineering and application of glycosidase derived biocatalysts in the study of mycothiol pathway enzymes

by

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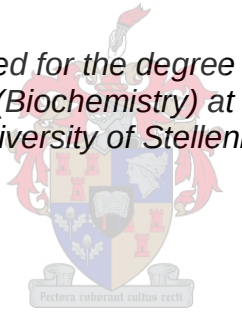

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We all lose and fall, winners rise and conquer
~ Anonymous ~

Abstract

Glycosides are complex carbohydrates that are involved in essential cellular and molecular biological processes within all living organisms. In addition, certain glycosides have anti-cancer, antioxidant, anti-inflammatory, antibacterial, antiviral, antiparasitic and antifungal activities. The functions of glycosides in biological processes and in biomedicine have led to a high demand for these organic molecules. However, the study of glycosides is hindered by the practical challenges in generating these compounds synthetically. This is even more true in the case of glycosides bound by means of α-glycosidic bonds, as most of the available synthetic methods promote the formation of β-glycosides. Methods that form α-glycosides are either low yielding or also promotes β-glycoside formation, resulting in the formation of mixtures that are challenging to separate. During the course of this study novel α-thioglycoligases derived from a CAZy family GH89 α-*N*-acetylglucosaminidase were prepared and characterized for their ability to form α-glycosides through biotransformation. The utility of the new biocatalysts was showcased by preparing several α-GlcNAc-based glycosides of biomedical and chemical interest. The products were purified or modified further through chemical transformations (such as “Click”-cycloaddition) and subsequently tested as potential small molecules chaperones for the treatment of Sanfilippo syndrome and/or as alternative substrates for the MshB, a mycothiol biosynthetic enzyme that has been identified as a potential target for development of new antituberculosis agents. Through the discovery of these molecules and the development of a new continuous deacetylase enzyme assay the findings of this study will significantly strengthen our ability to mobilise α-GlcNAc-based glycosides as part of ongoing research efforts in these fields.

Opsomming

Glikosiede is komplekse koolhidrate wat betrokke is by noodsaaklike sellulêre en molekulêre biologiese prosesse binne alle lewende organismes. Daarbenewens het sommige glikosiede ook anti-kanker, anti-oksidant, anti-inflammatoriese, antibakteriële, antivirale, antiparasitiese en antifungale aktiwiteite. Die funksies van glikosiede in biologiese prosesse en in die biomediese vakgebied het gelei tot 'n groot aanvraag vir hierdie organiese molekules. Die studie van glikosiede word egter verhinder deur die praktiese uitdagings om hierdie verbindings sinteties te berei. Dit is selfs meer waar in die geval van glikosiede gebind deur middel van α-glikosidiese bindings, aangesien die meeste van die beskikbare sintetiese metodes die vorming van β-glikosiede bevorder. Metodes wat α-glikosiede vorm het óf 'n lae opbrengs óf bevorder ook β-glikosied-vorming, wat lei tot die vorming van mengsels wat uitdagend is om te skei. Gedurende die verloop van hierdie studie is nuwe α-tioglikoligases wat afgelei is van 'n CAZy familie GH89 α-N-acetylglucosaminidase berei en gekarakteriseer vir hul vermoë om α-glikosiede vorm deur biotransformasie. Die nut van die nuwe biokataliste is ten toon gestel deur die voorbereiding verskeie α-GlcNAc-gebaseerde glikosiede van biomediese en chemiese belang. Die produkte is gesuiwer of verder verander deur chemiese transformasies (soos "Click"-sikloaddisie reaksies) en daarna getoets as potensiële klein molekule chaperones vir die behandeling van Sanfilippo sindroom en/of as alternatiewe substrate vir MshB, 'n mikotiol biosintetiese ensiem wat geïdentifiseer is as 'n potensiële teiken vir die ontwikkeling van nuwe antituberkulose middels. Deur die ontdekking van hierdie molekules en die ontwikkeling van 'n nuwe deurlopende deasetieleringsensiem-essaï sal die bevindinge van hierdie studie ons vermoë om α-GlcNAc-gebaseerde glikosiede te mobiliseer as deel van volgehoue navorsing op hierdie gebied aansienlik versterk.

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List of Abbreviations:

λ_{em}	emission wavelength
λ_{ex}	excitation wavelength
^{1}H NMR	proton nuclear magnetic resonance spectroscopy
1-L-Ins-1-P	1-L-myo-inositol-1-phosphate
6xHis-tag	$(His)_6$ peptide tag
AcCoA	acetyl-coenzyme A
AcCySmB	*N*-acetylcysteine-monobromobimane conjugate
AccQ-Fluor™	6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate
ACS	acetyl-CoA synthetase
ADP	adenosine 5'-diphosphate
AK	acetate kinase
Ala	Alanine
AMP	adenine 5'-monophosphate
ATP	adenine 5'-triphosphate
Bca	bacillithiol S-conjugate amidase
$BF_3.Et_2O$	boron trifluoride diethyl etherate
BSH	bacillithiol
BST	bacillithiol S-transferase
CAT	cysteinyl-S-conjugate acetyltransferase
CD	circular dichrosim
CH_2Cl_2	dichloromethane
CoA	coenzyme A
*Cp*GH89	α-*N*-acetylglucosaminidase of *Clostridium perfringens*

CS	Citrate synthetase
CSA	5'-*O*-[*N*-L-cysteinyl)sulfamonyl]adenosine
CT	C-terminal
C. perfringens	*Clostridium perfringens*
Cys	cysteine
Cys-GlcN-Ins	1-D-*myo*-inositol-2-(L-cysteinyl)-amido-2-deoxy-α-D-glucopyranoside
D_2O	Deuterium oxide
DNA	deoxyribonucleic acid
DMSO	dimethylsulfoxide
DMPU	1,3-dimethyl-3,4,5,6-tetra-hydro-2(1H)-pyrimidinone
DNTB	5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent)
2,4DNP-α-GlcNAc	2,4-dinitrophenyl α-*N*-acetyl-D-glucosaminide
DTT	dithiothreitol
E. coli	*Escherichia coli*
ESI-MS	electron spray ionization mass spectroscopy
FSA	fluorescamine
GlcNAc	*N*-acetyl-glucosamine
GlcNAc-SBn	benzyl-2-acetamido-2-deoxy-1-thio-3,4,6-tri-*O*-acetyl-α-D-glucopyranose
GlcNAc-SDNP	2,4-dinitrophenyl 2-acetamido-2-deoxy-1-thio-α-D glucopyranose
GlcNAc-Ins	1-D-myo-inosityl-2-acetamido-2-deoxy-α-D-glycopyranoside
GlcNAc-Ins-3-P	1-D-myo-inosityl-2-acetamido-2-deoxy-α-D-glycopyranoside-3-phosphate
GlcNAc-Mal	2S-2-2-acetamido-2-deoxy-α-D-glucopyranosyl-oxy-succinic acid
GlcN	glucosamine

GlcN-Ins	1-D-myo-inosityl-2-deoxy-α-D-glycopyranoside
GlcN-Mal	2-deoxy-α-D-glucopyranosyl-oxy-succinic acid
Glu	glutamic acid
Gly	glycine
GNAT	GCN5-related *N*-acetlytransferase
h	hour
H_2O	water
HCl	hydrochloric acid
HEPES	*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid
His	histidine
hrs.	hours
HPLC	high pressure liquid chromatography
HPAEC-PAD	High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection
H_2SO_4	sulfuric acid
Ile	isoleucine
IPTG	isopropyl-β-D-thiogalactoside
IMAC	immobilized metal affinity chromatography
Ins	*myo*-inositol
LCMS	liquid chromatography mass spectrometry
LDH	lactate dehydrogenase
L-Ins-1P(I1P)	L-*myo*-inositol-1-phosphate
LMW	low molecular weight
Lys (L)	lysine
mBBr	monobromobimane

Mca	mycothiol-S-conjugate amidase
MDR-TB	multidrug-resistant *M. tuberculosis*
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
min	minute
M. tuberculosis	*Mycobacterium tuberculosis*
Mtr	mycothiol disulfide reductase
MSH	mycothiol
MsmB	bimane derivative
M. smegmatis	*Mycobacterium smegmatis*
MS	mass spectrometry
MscR	mycothiol-*S*-nitrosoreductase/-formaldehyde dehydrogenase
MST	mycothiol S-transferase
MSSM	mycothiol disulfide
M_r	relative molecular mass
NaCl	sodium chloride / salt / brine
N-AcCys	*N*-acetyl-cysteine
NADH	reduced nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
NaN_3	sodium azide
NDA	naphthalene-2,3-dicarboxaldehyde
NMR	nuclear magnetic resonance spectroscopy
NT	N-terminal
NTB	2-nitro-5-thiobenzoate

OD_{600}	optical density at 600nm
*2*NP-α-GlcNAc	*o*-nitrophenyl α-*N*-acetyl- D -glucosaminide
*4*NP-α-GlcNAc	*p*-nitrophenyl α-*N*-acetyl-D-glucosaminide
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate
PPi	pyrophosphate
PK	pyruvate kinase
PTA	phosphotransacetylase
RE	restriction enzyme
R_f	retention factor
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
RS	reactive species
RT	room temperature
s	seconds
S. aureus	*Staphylococcus aureus*
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Ser	serine
SH	thiol group
SOE PCR	splicing by overlap extension polymerase chain reaction
TAE	triethanolamine
TB	pulmonary tuberculosis
TCEP	*tris*(2-carboxyethyl)phosphine
TGL	thioglycoligases

TLC	thin layer chromatography
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
Tyr	tyrosine
U	units (enzyme concentration)
UDP	uridine-diphosphate
UDP-GlcNAc	uridine-diphosphate-*N*-acetyl-glucosamine
UV	ultraviolet
$^{w}/_{w}$	mass fraction – weight/weight
$^{w}/_{v}$	mass fraction – weight/volume
Val	valine
$^{v}/_{v}$	volume fraction – volume/volume
XDR-TB	extensively drug-resistant *M. tuberculosis*

Chapter 1: Introduction

1.1 Introduction

Glycosides are complex carbohydrates that are comprised of a sugar moiety linked either to another sugar moiety or a non-sugar moiety through a glycosidic bond [1-3]. The sugar moiety is referred to as a glycone, while the non-sugar part is called an aglycone. An example is shown in Figure 1.1. The glycone can comprise a single sugar (monosaccharide) or multiple sugars, including disaccharides (di = 2), oligosaccharides (oligo = few) or polysaccharides (poly = many). As the names indicate, disaccharides contain two monosaccharide units, oligosaccharides consist of three to ten units and polysaccharides have more than ten units [1, 4-6]. The aglycone moiety can either be an alcohol, aryl group, lipid, protein, nucleic acids etc. [2, 4, 7, 8].

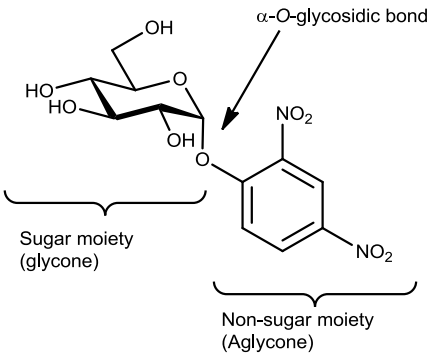


Fig. 1.1: A structure of a chromogenic glycoside (2,4-dinitrophenyl-α-D-glucopyranose) consisting of a glycone (glucopyranose) linked to aglycone (2,4-dinitrophenol) through an α-*O*-glycosidic bond

Glycosides have many essential functions within living organisms. These organic molecules are classified based on the structures of the glycone moiety (or moieties) and the aglycone moiety (chromogenic, cyanogenic, etc.), the configuration of the glycosidic bond (α or β) and the atom involved in the glycosidic bond linkage, that is, carbon, oxygen, nitrogen and sulphur. It is based on these atoms that glycosides are

referred to as *C*-, *O*-, *N*- or *S*-glycosides. The most abundant type of glycoside linkage both naturally and synthetically is the *O*-glycosidic bond, which leads to the formation of *O*-glycosides [3]. Glycosides are a very diverse and complicated family of compounds owing to the glycone and aglycone moieties and are involved in numerous essential biological and biomedicinal functions, as detailed below [9-11].

1.2 Function of glycoside

Glycosides play several essential biological roles within the cellular and molecular processes of living organisms [2, 10-16]. The cellular process includes cellular transport, adhesion, cell-to-cell recognition and the molecular processes including, but not limited to, embryogenesis, fertilisation, neuronal development and proliferation of cells. In biomedicine, glycosides are involved in facilitating the mode of action of various drugs through stabilization of protein folding and by aiding the active transmembrane transport system; or they act as inhibitory compounds [2, 10, 17-21]. In most of these instances it is the glycone moiety that confers the activity, as is the case of daunomycin and erythromycin. In fact, the glycone moiety facilitates the physical, chemical and biological properties of its aglycone moiety. In addition, it regulates the pharmacokinetic properties of how the drug is **a**bsorbed, **d**istributed, **m**etabolised and **e**xcreted (ADME) by the host system [2, 10, 21]. Certain glycosides have anti-cancer, antioxidant, anti-inflammatory, antibacterial, antiviral, antiparasitic and antifungal activities [10, 17-19, 21-24]. The ever first effective antibiotic against *Mycobacterium tuberculosis* (*M. tuberculosis*), the main causative agent of Tuberculosis (TB) was Streptomycin (Figure 1.2), an aminoglycoside isolated from a soil bacterium, the Actinobacterium *Streptomyces griseus* [17, 22-24]. Other antibiotic glycosides include aminoglycosides such kanamycin, gentamicin, bleomycin among others, all of which are used to treat infections by pathogenic Gram-negative bacteria such as *Pseudomonas* and *Salmonella* species etc. In addition, infections by pathogenic Gram-positive bacteria such *Staphylococcus aureus, Streptococcus pneumonia* etc., genetic disorders and human immunodeficiency virus infections are also treated by compounds that include glycosides [10, 17, 22-26].

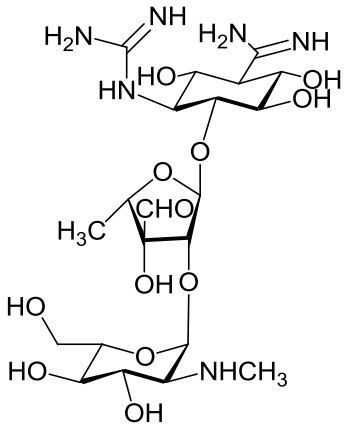


Fig. 1.2: The structure of Streptomycin

The essential roles of glycosides in several fundamental biological processes and the wide use thereof in biomedicine have led to a high demand for certain glycosides, and consequently also the development of various ways to produce them synthetically. This is because the extraction of glycosides from plant or soil bacteria normally results in low yield due to the number of purification steps required to obtain a pure final product. However, the chemical synthesis of glycosides often encounters difficulties in generating the bond with the correct anomeric configuration, and linked to the correct substituent of the glycone. Therefore, while glycosides can be synthesized either chemically and/or enzymatically, the interest in the latter, more specific, method has been receiving increasing interest.

This project focused on the generation of α-*N*-acetyl-glucosamine (GlcNAc)-based glycosides of biomedical and chemical interest through enzymatic synthesis, particularly since these glycosides are known to be produced in low yields [8, 27, 28]. In the next section the methods currently available for the chemical and enzymatic synthesis of glycosides are reviewed, the motivation for the generation of α-GlcNAc-based glycosides is provided, and the problem of generating such glycosides is stated. This finally leads to the statement of the objectives of this project.

1.3 Synthetic preparation of glycosides

Glycosides are synthesized through a process known as glycosylation that involves the formation of a glycosidic bond [2, 3, 5, 7, 8]. Whether by chemical or enzymatic methods, glycosylation has four basic requirements: (1) the use of a glycosyl donor that contains a good leaving group (LG) at the anomeric centre; (2) an acceptor molecule with a suitable nucleophilic group, which can be either a sugar moiety or a non-sugar moiety; (3) controlled environmental conditions and; (4) a suitable promoter/catalyst. (Figure 1.3) [7, 9]. The formations of glycosidic bonds by either the chemical or enzymatic route both have their benefits and shortcomings, as outlined below.

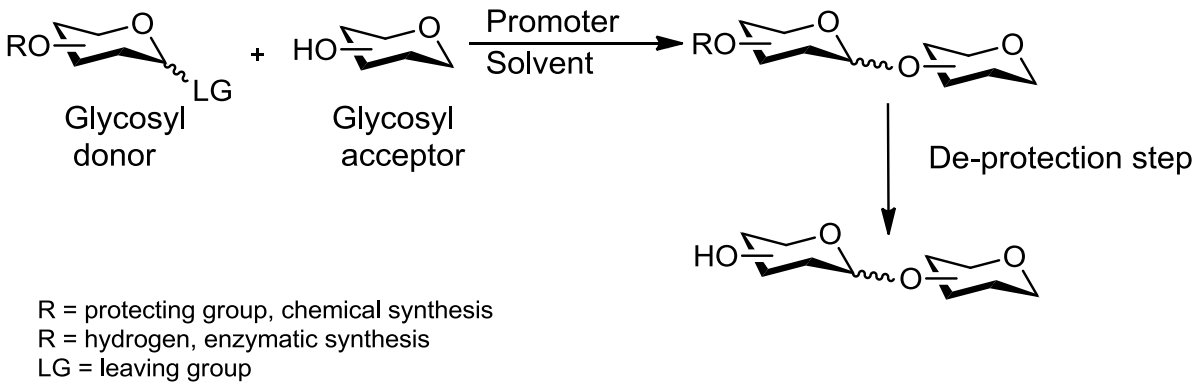


Fig. 1.3: General mechanism of glycoside bond formation between two sugar moieties, one acting as donor, and the other acceptor. Glycoside bond formation through enzymatic synthesis does not require protecting groups, hence the final de-protecting step is not needed [3, 9].

1.3.1 The chemical synthesis of glycosides

The groundwork of synthetic carbohydrate chemistry was laid by Emil Fisher in the late 1800's [3, 29-31]. It is reported that the first glycosides chemically synthesized were aryl glycosides by Michael in 1879 [2, 7]. This was followed by generation of glycosides through acid-catalysed alcoholysis of monosaccharides in 1893 by Fisher [2, 7]. In the early 1900s, Koenings and Knorr developed other chemical methods for the synthesis of glycosides based on the synthesis of Michael [2, 3, 5, 7]. In modern times, Ray Lemieux is considered to be one of the foremost contributors to the chemical synthesis of carbohydrates [3, 32]. He was the first person to chemically

synthesize sucrose and he developed key glycosylation methods, such as *in situ anomerisation* [3]. Since then, several chemical and enzymatic glycosylation methods have been established which are utilised to generate disaccharides, oligosaccharides, polysaccharides, glyco-conjugates and glycosaminoglycans molecules [5, 9, 33].

Chemical formation of a glycosidic bond includes vigilant selection of suitable promoter(s), correct protecting groups for both the glycosyl acceptor and donor, proper selection of a leaving group at the anomeric centre of the donor in order to give rise to the correct regio- and stereoselectivity [3, 5, 7, 9]. In addition, it also includes careful removal of the protecting group without destabilizing the final product. The general mechanism follows a SN_1-like mechanism and SN_2-like displacement which can occur through different ion intermediates such as oxocarbenium ion, acetoxonium ion or oxazoline [3, 8, 9, 13, 34]. It is through these intermediates that the glycosidic bond is formed and its anomeric configuration is fixed.

1.3.1.1 The chemical formation of β- and α-glycosides

Glycosidic bond formation is highly influenced by the choice of the donor and acceptor molecules, the promoter/catalyst used, the temperature of the reaction, the solvent used and the choice of protecting groups [3, 5, 7, 9, 34-36]. Furthermore, the sequence in which the reagents are added could also influence the anomeric configuration of the formed glycoside. However, the most prominent influence of the anomeric configuration is exerted by the group attached at the second carbon (C2) of the gycosyl donor [9, 36]. The first step of glycosidic bond formation involves the loss of the LG; this activates the glycosyl donor through formation of an oxocarbenium ion intermediate (Figure 1.4) [13]. The departure of the leaving group is also assisted by lone pairs of the ring oxygen, which also stabilised the oxocarbenium ion intermediate through resonance. This intermediate can subsequently be attacked either from the bottom or top by the nucleophile of the glycosyl acceptor to form the glycoside bond.

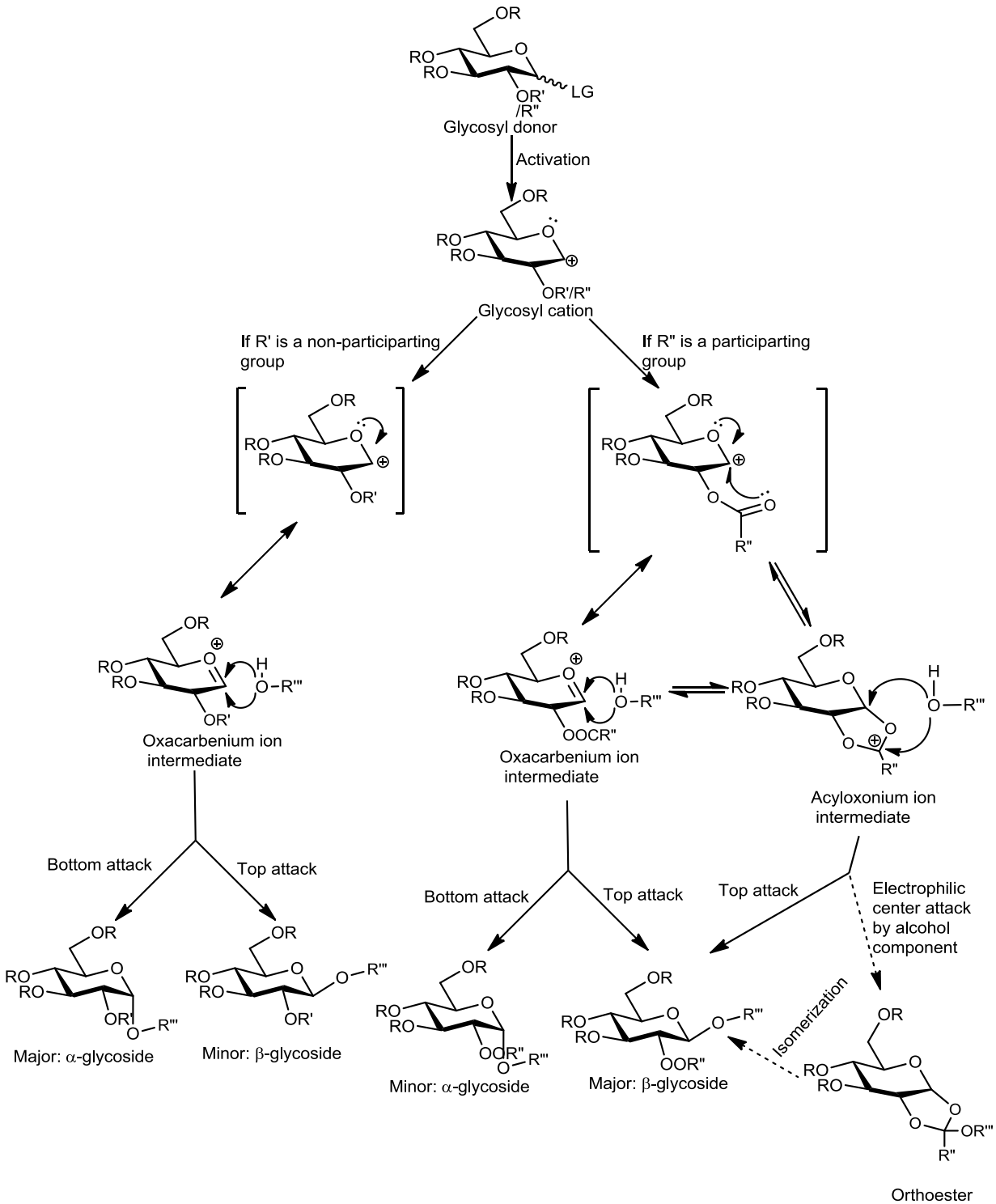


Fig. 1.4: The chemical mechanism of glycosidic bond formation. On the left the formation of glycosidic bonds from a glycosyl donor without a participating group at C2 is shown, while glycoside bond formation from a glycosyl donor with a participating group at C2 is shown on the right. R, protecting group; R', non-participating groups such benzyl, azido etc. R'', participating groups such alkyl, aryl etc. and R'''as glycosyl acceptor which can be a sugar moiety or aglycone [3, 9, 34].

The attack of the intermediate, that is either top or bottom, is highly dependent on the nature of the group at C2, specifically whether it is a participating or non-participating group. For example, when a glycosyl donor containing a non-participating functional group such as azide, benzyl etc. at C2 is used as a donor (together with careful selection of other factors), the formation of the α-anomeric configuration is promoted over the β-configuration (Figure 1.4). The α-glycosides form when the nucleophile of the glycosyl acceptor attacks the oxocarbenium ion intermediate from the bottom, while the attack from the top would lead to formation of the β-anomer. The attack from the bottom of the oxocarbenium ion intermediate is thermodynamically favoured as a result of the anomeric effect at the anomeric carbon which stabilizes the formation of α-glycosides since no other group it is participating within the oxocarbenium ion intermediate [4, 5, 8, 9].

In a situation where a glycosyl donor contains a participating functional group (such as alkyl, aryl, acyl, ester groups) at C2, an acyloxonium ion intermediate is generated from the oxocarbenium ion intermediate (Figure 1.4) [3, 8, 9, 35]. After the activation step the oxocarbenium ion intermediate is stabilised by the carbonyl oxygen of the participating group at position C2 which results in the formation of acyloxonium ion intermediate. Once the acyloxonium ion intermediate is formed, its dioxolane ring is attacked by the nucleophile of the glycosyl acceptor either from the top or the bottom to form a β- or α-glycosidic bond, respectively. Acyloxonium ion intermediates preferentially promote the formation of β-glycosides over the α-counterparts due to the effect of the participating group. Attack of the carbocation resonance form of the acyloxonium ion by an alcohol nucleophile under basic or neutral conditions produces a by-product known as an orthoester. Orthoesters can eventually be converted into β-glycosides through isomerization in most, but not all, cases [3, 34].

In the case of *N*-acetyl-aminoglycoside synthesis a glycosyl donor with *N*-acetyl group at the C2 is used as either a protecting or permanent functional group. This reaction occurs through a different intermediate known as an oxazoline which is also formed via the oxocarbenium ion intermediate [8]. The oxazoline intermediate (Figure 1.5) is attacked in the same way as the acyloxonium ion by the nucleophilic glycosyl acceptor, and consequently they mostly also preferentially promote the formation of β-glycosides. However, due to the increased stability of the oxazoline

intermediate compared to the acyloxonium ion these reactions usually have long reaction times with low yields.

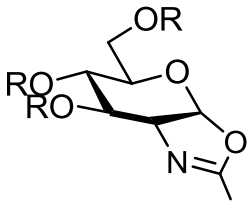
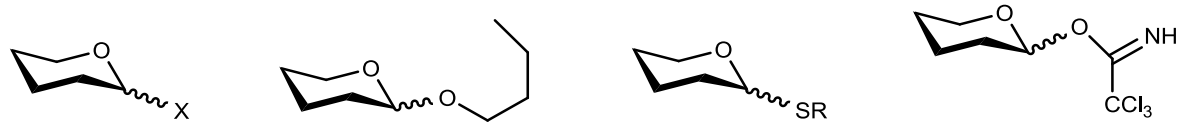


Fig. 1.5: The oxazoline intermediate [8]

1.3.1.2 Chemical methods used to generate glycosidic bonds

Chemical glycosylation methods are generally evaluated based on the amount of reagents they require, the yield of the product, whether they are stereo-selective and whether they allows for large scale production [7]. To date a number of chemical methods have been established for the formation of *O*-glycosidic bonds and some of them can also be used to synthesise *N*-, *S*- or *C*-glycosidic bonds. The chemical methods for the formation of *O*-glycosidic bonds include Michael, Fischer, Helferich, Koenigs-Knorr, Schmidt/trichloroacetimidates, Sulfure and Armed-Disarmed reactions method amongst others [2, 3, 7, 37, 38]. The Michael, Fischer, and Koenigs-Knorr methods were the first established glycosylation methods, although currently the Koenigs-Knorr, Schmidt/trichloroacetimidates, sulfure and Armed-Disarmed methods are the most frequently applied chemical glycosylation methods. These methods are distinguished based on the nature of the glycosyl donors that are used (Figure 1.6).



Glycosyl halides (X = Bromide, chloride etc) | *n*-Pentenyl glycosides | Thiolycosides | Glycosyl trichloroacetimidates

Fig. 1.6: The structure of the glycosyl donors used in the most popular chemical methods for formation of the O-glycosidic bond. Glycosyl halides are used as donor during Koenigs-Knorr, while Schmidt/trichloroacetimidates, sulfur and Armed-Disarmed methods use glycosyl trichloroacetimidates, thioglycosides and *n*-pentenyl glycosides as donors, respectively.

Koenigs-Knorr uses glycosyl halides as donor, while Schmidt/trichloroacetimidates, sulfur and Armed-Disarmed methods use glycosyl trichloroacetimidates, thioglycosides and *n*-pentenyl glycosides as donors, respectively. In addition, they also utilise different promoters, which is a component added to a reaction to increase the catalyst reactivity and activity. For example, the Koenigs-Knorr method uses silver, mercury and other heavy metal salts as promoters, while the sulphur methods use promoters such *N*-iodosuccinimide or iodinium dicollodine. Koenigs-Knorr is the most used method for generation of the *O*-glycosidic bond and was established in 1901 by Wilhelm Koenigs and Eduard Knorr [3]. This method mostly promotes the formation of the β-glycosidic bond configuration as a result of the donor and promoter used. Figure 1.7 provides an example of glycosidic bond formation through the Koenigs-Knorr method. Koenigs-Knorr method is also used for the preparation of *n*-pentenyl glycosides which are used as glycosyl donor in the Armed-Disarmed method to synthesise large oligosaccharides such pseudo-tetrasaccharide. The other methods also occur in the similar way as the Koenigs-Knorr method and also result mostly in the formation β-glycosidic bond confirmation.

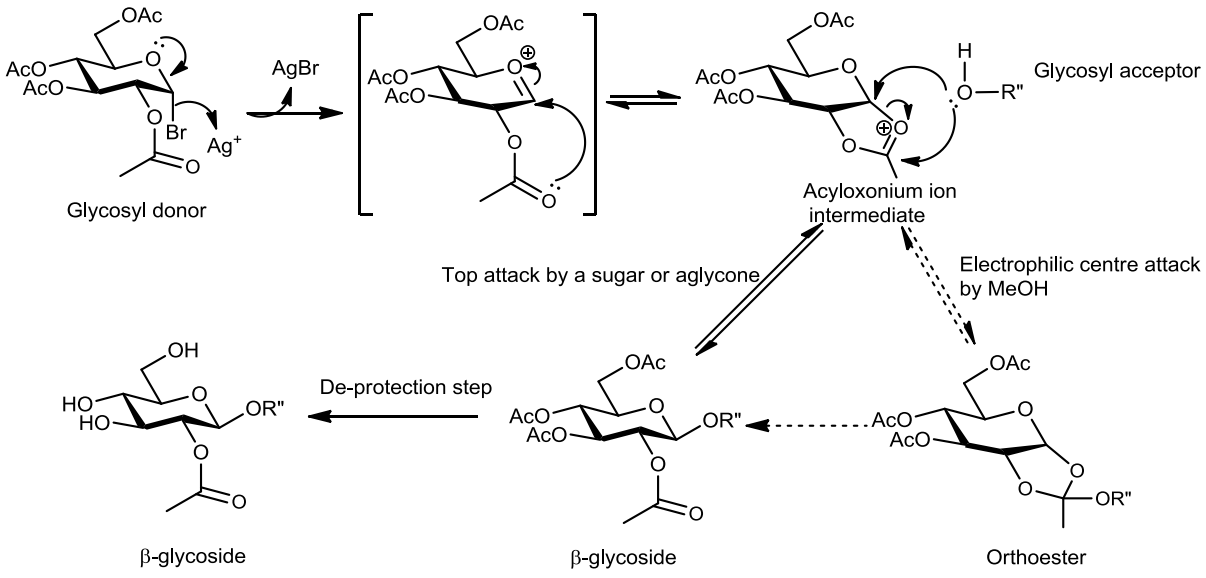


Fig. 1.7: The formation of glycosidic bond through Koenigs-Knorr method with an acetylated glycosyl bromide containing a participating group as the donor. The Koenigs-Knorr method mostly results in the formation of the β-glycosidic bond configuration due to the nature of the glycosyl donor [3, 5, 37].

In addition to these methods, Lemieux and co-workers developed a kinetically controlled glycosylation method in 1975 (known as *in situ* anomerization) for the generation of α-glycosidic bonds [39]. In this method, generation of the glycosidic bond is highly depended on the anomeric effect at the anomeric centre, which destabilises the leaving group of the glycosyl donor. This effect was discovered by Raymond Lemieux and is highly influenced by non-participating functional group of the C2 and the electronegativity of the anomeric substituent: the more electro-negative the substituent at the anomeric is, the larger the anomeric effect [3, 5, 6]. The anomeric effect occurs as a result of the dipole-dipole interaction next to the anomeric centre and stereoelectronic effect at the anomeric centre. Two dipole interactions occur near the anomeric centre which influences the anomeric effect. The first interaction dipole is that of the two non-bonding electron pairs of the endocyclic carbohydrate ring oxygen, which generates a dipole in the exocyclic direction. The second dipole occurs as a result of the polarized bond between the anomeric carbon atoms and the exocyclic heteatom. The interaction of the two dipoles in β-anomers is parallel to each other facing the same direction as the anomeric carbon atom exocyclic heteratom bond of which is chemically energetically poor and unstable (Figure 1.8a). In the case of α-anomers the dipoles are antiparallel facing away from each other resulting in the bond that is stable and chemically energetically favoured (Figure 1.8b). Anomeric effect promotes the formation of the α-glycosidic bond. The formation of the α-glycosidic bond through *in situ* anomerization method also requires highly controlled and careful selection of promoter, solvent, glycosyl donor and leaving group in order to avoid formation β-glycosidic bond.

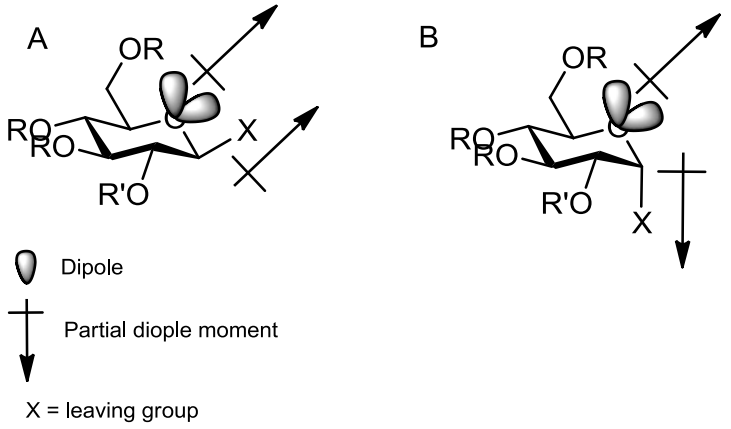


Fig. 1.8: The anomeric effect of the β-(A) and α-(B) glycosidic bond formation [3, 6]

1.3.1.3 Shortcomings of chemical glycosylation methods

The biggest shortcoming of the various chemical glycosylation methods that have been developed to date is the fact that they nearly all favour the formation of β- over α-glycosides. Apart from this, some of the methods also use toxic chemicals to the human system. In most cases chemical glycosylation methods also require the protection and de-protection of the various substituents in order to get the correct regio- and stereo-selectivity. This can lead to multi-step syntheses with low yields.

The formation of α-glycosidic bonds by chemical methods still remains a big challenge. In addition, chemical methods for α-glycosides often also lead to the formation of β-glycosides as side-products during the reaction; this in turn creates another challenge as the required α-glycoside has to be purified from the mixture. The formation of GlcNAc-based glycosides with the α-configuration in particular still remains a significant challenge as a result of the acetyl group at C2 which participates in the reaction during the glycosidic bond formation, giving rise to the preferential formation of β-glycosides over their α-counterparts. As such, new chemical methods that will promote the formation of α-GlcNAc glycosides still need to be developed.

1.3.2 The biocatalytic synthesis of glycosides

Apart from the various chemical methods to prepare glycosides, several enzymes have also been applied in the synthesis of these compounds, i.e. through a biocatalytic process [3, 5, 40, 41]. For the enzymatic synthesis of glycosides enzymes such as glycosyltransferases, glycosidases or glycosidases modified at their catalytic residues are usually used. There are various requirements for such enzymes to be used in biocatalysis. First, the enzyme in question needs to be able to form the desired glycoside, but not hydrolyse or otherwise transform the final product. Second, the enzyme must be stable under conditions that may be very different from its natural physiological environment, and third, the conditions under which the enzyme functions optimally should be known. Lastly, information regarding the mechanism of the enzyme that is to be used must be available. This helps to know what kind of donors and acceptors can be used.

The current biocatalytic methods available for the synthesis of glycosides mostly promote the formation of the *O*-glycosidic bonds, although one type of modified

glycosidase also promotes *S*-glycosidic bond formation. The configuration of the glycosidic bond (α- or β-) is highly dependent on the type of enzyme used, as well as on the glycosyl donor to some extent. Enzymatic glycosylation usually occurs under mild conditions without the requirement for protecting groups. In the following sections the various enzymes that are used to generate glycosidic bonds through biocatalytic methods are briefly discussed.

1.3.2.1 Glycosyltransferases for biocatalytic glycoside synthesis

Glycosyltransferases are anabolic enzymes that generate the formation of the *O*-glycosidic bond between a sugar moiety and either another sugar moiety or an aglycone [42-46]. If the aglycone is a lipid, a glycolipid gets produced while a glycoprotein is the product if it is a protein. Glycosyltransferases are classified as either inverting or retaining based on the catalytic mechanism they use for the glycosidic bond formation, and often can be identified based on their primary sequence. In addition, they are also classified into clans depending on their fold: glycosyltransferases-A and glycosyltransferases-B. More than 90 families of glycosyltransferases have been characterised to date and their information is available on the Carbohydrate-Active enZYmes (CAZY) database (http://www.cazy.org). These enzymes utilises inverting and retaining mechanism for the formation of glycosidic bond [43, 44, 46]. The inverting mechanisms occur in a way similar to that of glycosidases, which will be discussed in more detail below. The only difference is that the *inverting* glycosyltransferases-A requires Mn^{2+} which acts as acid catalyst [44, 47]. On the other hand, the catalytic mechanism of *retaining* glycosyltransferases is not yet fully understood [43, 46]. It proposed that some retaining glycosyltransferases use a double-displacement mechanism while other use a single-displacement mechanism, with the latter one having more support. Glycosyltransferases utilise activated donors such as nucleoside mono/di-phosphosugars and lipid phosphosugars among others for the formation of glycosides [41, 48]. More than 60 glycosyltransferases—some of which are commercially available—have been characterized for the chemo-enzymatic synthesis of glycosides [41]. Nonetheless, the utilisation of such enzymes is hindered by the fact that most glycosyltransferases are not stable once out of their natural physiological environment, and are subject to feedback inhibition. In addition,

the glycosyl donors required by these enzymes are too expensive to allow for their use on preparative scale.

1.3.2.2 Glycosidases for biocatalytic glycoside synthesis

Glycosidases are hydrolytic enzymes that normally cleave the glycosidic bonds found in oligosaccharides, polysaccharides, glycoproteins and glycolipids [49, 50]. These enzymes are divided into various families depending on their primary sequence, their structural fold and the reaction mechanism they use for hydrolysis of the glycosidic bond [51-54]. Glycosidases have many different structures, which is believed to be due to the diversity of both their natural substrates and the evolution of the active site residues that is used to cleave the bond. More than 100 different families have been identified, with their taxonomy regularly being updated at http://www.cazy.org. Glycosidases are classified based on the anomeric configuration of the bond that they cleave (i.e. as α- or β-glycosidases) and on the mechanism used to cleave the bond (i.e. whether they are inverting- or retaining-glycosidases) [55]. In addition, they are also classified as exo-glycosidases or endo-glycosidases depending on the position of the bond that is cleaved [49]. Endo-acting glycosidases cleave any glycosidic bond except those attaching the terminal saccharide units in chains, while exo-acting glycosidases act on the bonds attaching these terminal units (Figure 1.9).

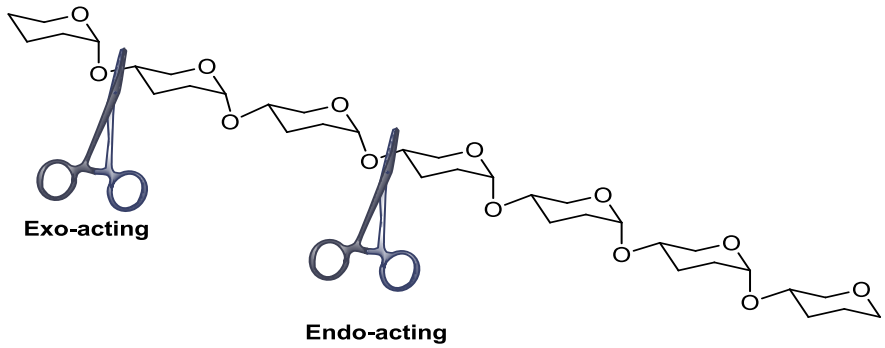


Fig. 1.9: Exo- and endo-acting glycosidases. The exo-acting enzyme in this example gives rise to a monosaccharide and oligosaccharide, while the endo-acting glycosidase results in two oligosaccharides: one consisting of three subunits and the other of four subunits.

Whether glycosidases are inverting or retaining, in both cases the mechanism involves an oxocarbenium ion-like transition state and a pair of carboxylic acids located on opposite sides of the enzyme's active site [54, 56, 57]. However, they differ in that enzymes with a retaining catalytic mechanism depend on a double displacement reaction, while those with an inverting mechanism make use of a single displacement. The final product of a retaining glycosidase has the same anomeric configuration as the substrate, while the opposite is true for inverting enzymes (Figure 1.10). In addition, the distance between the catalytic residues are different between the two mechanisms; in the case of inverting enzymes these residues are often very far apart from each other, thus giving space for water (or other molecule) to intervene in the process. This stands in contrast to enzymes with a retaining mechanism, where the catalytic residues are much closer [56]. For example, the distance between the residues of an inverting enzyme is 9.0 ± 1.0 and 9.5 Å for α and β-glycosidases respectively, while for enzymes with a retaining mechanism the active site acid and base residues are approximately 4.8 ± 0.5 and 5.3 ± 0.2 Å apart for α and β-glycosidases respectively.

During the inverting catalytic mechanism one carboxylic acid from the pair acts as an acid, while the second carboxylic acid acts as a base (being in its carboxylate form) [54, 56, 57]. The base deprotonates the incoming water molecule allowing it to attack the anomeric carbon. This releases the sugar or aglycone moiety attached at this centre, thereby facilitating the cleavage of glycosidic bond (Figure 1.10A).

In contrast, the retaining mechanism occurs through two steps, namely glycosylation and deglycosylation (Figure 1.10B). During the glycosylation step, one carboxylic acid acts as an acid while the other acts as a nucleophile. The nucleophile attacks the anomeric carbon directly to release the sugar or aglycone moiety attached at this centre (facilitated by its protonation by the acidic residue), and to form a covalent glycosyl-enzyme intermediate. Next the deglycosylation step occurs when the residue that had acted as an acid in the first step now acts as a base that deprotonates a water molecule to generate a nucleophile that attacks the anomeric carbon of the glycosyl-enzyme intermediate. This resolves the substrate-enzyme complex and promotes cleavage of the glycosidic bond, leading to formation of the final product that has the anomeric configuration as the substrate.

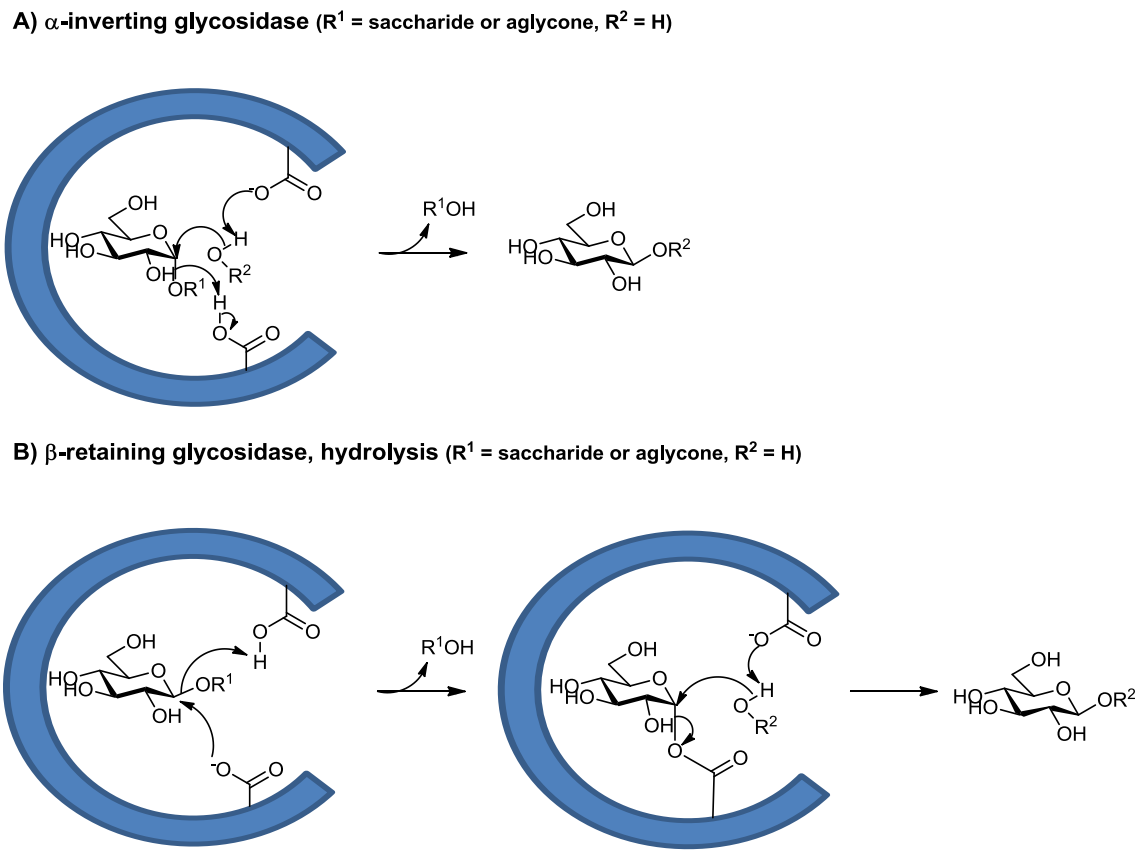


Fig. 1.10: Catalytic mechanisms of glycosidase [48]. **Panel A**, is the inverting mechanism and **panel B**, the retaining mechanism.

Glycosidases are known to not only promote hydrolysis, but to also promote glycosidic bond formation under specific conditions [58-60]. Glycosidase-mediated glycosidic bond formation occurs by one of two different mechanisms, namely by: reverse hydrolysis or by transglycosylation under retaining mechanism. The reverse hydrolysis reaction, also referred to as equilibrium control synthesis, depends on both concentration of monosaccharides as an acceptor and the presence of water. It is promoted by high concentration of monosaccharide acting as an acceptor and high concentration of organic solvents (80-90% v/v), such as acetonitrile, *tert* butanol etc. in relation to water [58]. The sugar moiety acting as an acceptor competes with water to act as the nucleophile that attacks the enzyme-substrate complex and, as a result of a low concentration of water and a high concentration of sugar acceptor, the sugar acceptor dominates leading to more glycosylation formation in relation to hydrolysis. Several glycosidases from different organisms have been used to

biosynthesis glycosides through the reverse hydrolysis reaction [58]. The transglycosylation mechanism also known as the kinetically controlled reaction depends on the rate activity of the substrate-enzyme complex formation, ratio between the rate activity of water concentration and rate activity sugar acceptor concentration to hydrolysis the substrate-enzyme complex [58-60]. This mechanism requires activated glycosyl donors such as nitrophenyl glycoside in order to promote fast and operationally irreversible cleaving of the donor. The reverse rate activity of the substrate-enzyme complex formation must be zero, that is, no reverse reaction of the substrate-enzyme complex formation. When the rate of attack by the sugar acceptor is higher than that by the water, transglycosylation is promoted and vice versa if the rate of the forward activity with water as nucleophile is higher than that of the acceptor.

The problem with the reverse hydrolysis or transglycosylation mechanism of glycosidase for the glycosidic bond formation is that the product formed in such a case also becomes a substrate for the hydrolytic activity of the enzyme; yields of glycosides formed in this way are usually low. To overcome this problem Withers and co-workers generated a new set of enzymes from glycosidase through mutation of one or both of the catalytic active site residues [56, 61]. Specifically, point mutation of the nucleophilic residue or the acid/base residue resulted in generation of glycosynthase and thioglycoligase enzymes, respectively. Double mutation, that is, the inactivation of both active site residues generated thioglycosynthases. More information on these non-natural enzymes acting as biocatalysts is provided in the next section.

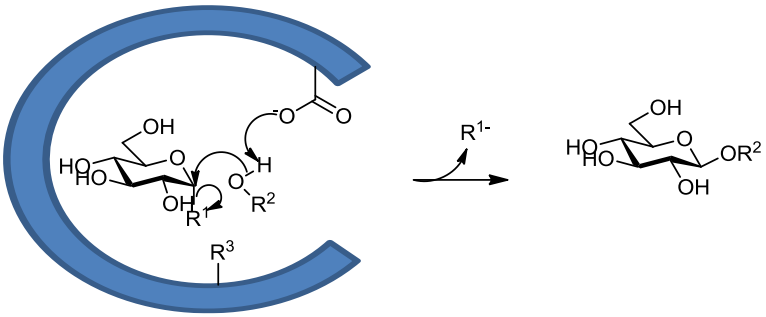
1.3.2.3 Glycosynthases as biocatalytic enzymes

Glycosynthases are mutated glycosidases at the nucleophilic catalytic active site, wherein the nucleophilic amino acid usually the glutamic acid or aspartic acid residue is changed into either a polar uncharged amino acid or non-polar aliphatic amino acid [40, 55, 56, 59, 61-63]. These enzymes are classified based on the glycosidase that they are generated from, and also on the anomeric configuration of the final product. For example, a glycosynthase generated from an exo-acting β-retaining glycosidase will be categorised as exo-acting β-glycosynthase. The first reported glycosynthase was derived from a retaining β-glucosidase enzyme from *Agrobacterium* sp. family 1 by Withers and co-workers in 1998 [61]. They mutated

the Glu345 residue of the β-glucosidase/galactosidase from *Agrobacterium* sp. into the non-polar aliphatic amino acid alanine. To generate the glycosidic bond activated α-glycosyl-fluoride was used as donor, with an aryl glycoside used as acceptor. Since then, many glycosidase from different families have been engineered using this approach to obtain glycosynthases [40, 59]. Nearly all of these employ the retaining mechanism, with only two inverting glycosidases having been converted to glycosynthases.

Glycosynthases require an activated glycosyl donor that contains a good leaving group (such as fluoride or dinitro-phenol), an external nucleophile (such as sodium azide/fluoride to mimic the mutated nucleophilic residue) and a glycosyl acceptor [40, 55, 56, 59, 61-63]. In addition, a glycosynthase derived from a β-glycosidase can act as both an inverting or retaining glycosynthase. In general, glycosynthases produced from thermophilic organisms utilises nitrophenyl glycosides as donors such 2-nitrophenyl-/4-nitrophenyl-N-acetyl-α-D-glucosaminide (2NP/4NP-GlcNAc) among other with either sodium azide or sodium formate as the external nucleophile. For glycosidases from mesophilic organisms donors with either fluoride and dinitrophenol leaving groups are mostly used, as these are more reactive but generally stable at standard reaction temperatures. The mechanisms of glycosynthases are shown in Figure 1.11. In the first step the glycosylation reaction occurs between the glycosyl donor and the external nucleophile to form a product complex which mimics the enzyme-substrate complex of the natural enzyme. This is followed by a deglycosylation step which occurs in the same way as it would have with the wild type glycosidase, forming a glycosidic bond which has the same anomeric configuration as the starting material [40, 55, 56, 59, 61-63]. Consequently, configuration of the product is highly dependent on the nature of the glycosyl donor. For example, when a glycosyl donor with an anomeric configuration opposite to that of the natural substrate of the parent enzyme is used, the glycosynthase forms a glycosidic bond that has the same anomeric configuration as that of the natural substrate (Figure 1.11A). In such a case, the glycosynthase acts an inverting enzyme, with the glycosidic bond formed in the same way as for glycosidases with an inverting mechanism. To act as a retaining glycosynthase the enzyme requires a glycosyl donor with the same anomeric configuration as that of the natural substrate of the parent enzyme (Figure 1.11B).

A: Inverting mechanism of glycosynthase
(R^1 = Fluoride, R^2 = glycone/ aglycone moiety and R^3 mutated nucleophilic active site)



B: Retaining mechanism of glycosynthase
(R^1 = good leaving group e.g. nitrophenol, R^2 = glycone/ aglycone moiety, R^3 mutated nucleophilic active site and R^4 = external nucleophile)

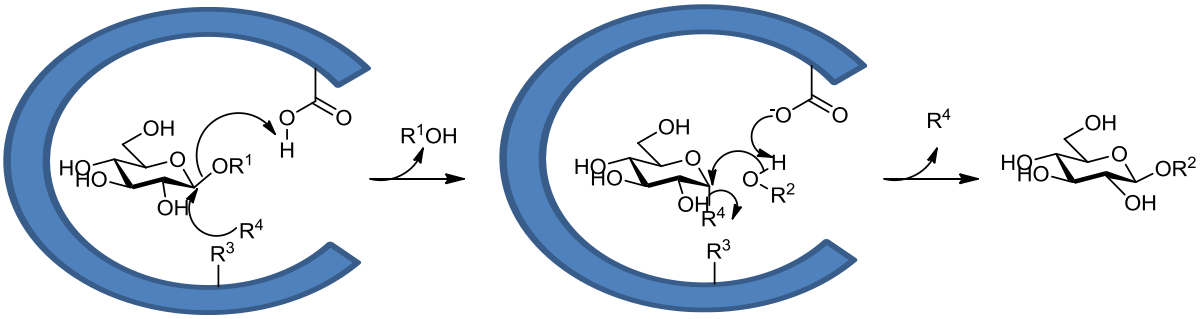


Fig. 1.11: The catalytic mechanism of β-glycosynthases. A) the inverting β-glycosynthase and B) retaining β-glycosynthase [59].

Ultimately both inverting and retaining β-glycosynthases generate a product with the same anomeric configuration to that of the natural substrate of the wild type enzyme. The only difference is that the retaining β-glycosynthase requires an external nucleophile and donor with the same anomeric configuration as the natural substrate, while the inverting β-glycosynthase needs a donor with an anomeric configuration that is opposite to that of the natural substrate.

1.3.2.4 Thioglycoligases as biocatalytic enzymes

Thioglycoligases are enzymes wherein acid/base catalytic active site usually the glutamic acid or aspartic acid residue is changed into either a polar uncharged amino acid or non-polar aliphatic amino acid [40, 49, 55, 59, 64, 65]. They promote the formation of *S*-glycosidic bond that is more resistant to enzymatic hydrolysis than *O*-glycosidic bonds. Moreover, thioglycosides have been found to be good inhibitory compounds for glycosidase enzymes [40, 65, 66]. Thioglycoligases are classified in the same way as glycosynthases. The first thioglycoligase was generated by Withers

and co-workers from a retaining β-glucosidase from *Agrobacterium sp.* [64, 66]. Since then several retaining glycosidases have been converted to thioglycoligases, only two of these being α-retaining glycosidases [40]. However, to date no thioglycoligase with an inverting mechanism has been generated.

For thioglycoligase activity the enzyme requires a glycosyl donor with a good leaving group that has the same anomeric configuration as the substrate of the parent enzyme, as well as a thio-compound as acceptor [40, 49, 55, 64, 65]. The reactions occur via a double displacement reaction, giving rise to the observed retention (Figure 1.12). The first step involves attack from the nucleophilic residue to form the enzyme-substrate complex. This complex is broken up when the thiolate of the acceptor reacts with the complex giving rise to a product in which the sugar of the donor is attached to the thiol acceptor with an *S*-glycosidic bond.

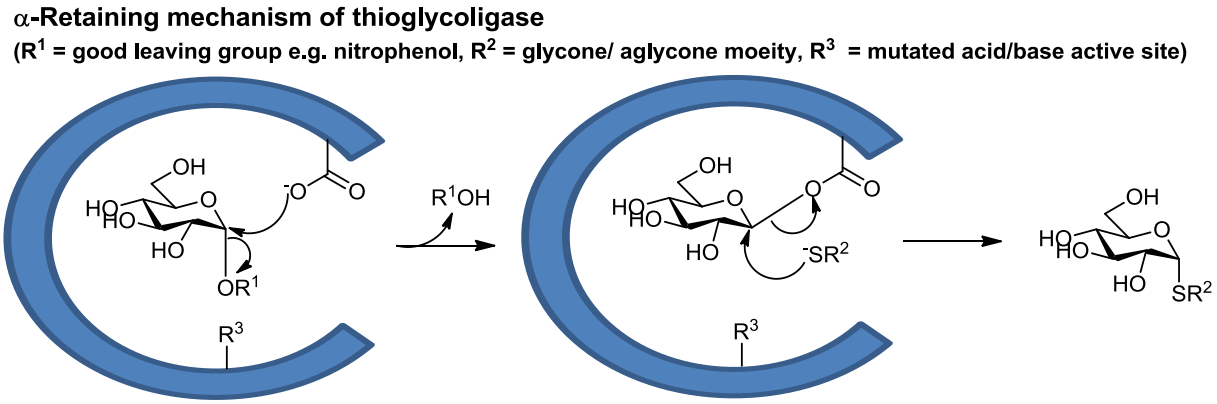


Fig. 1.12: The retaining catalytic mechanism of α-thioglycoligases

There is also one example of a thioglycosynthase that has been generated by Withers and co-workers from β-glucosidase of *Agrobacterium sp*. [67]. This is a glycosidase enzyme that is mutated at both catalytic residues, i.e. at both the acid/base and nucleophile residue sites (Figure 1.13). For this enzyme to function it requires a glycosyl fluoride as donor molecule and a thio-compound as an acceptor compound.

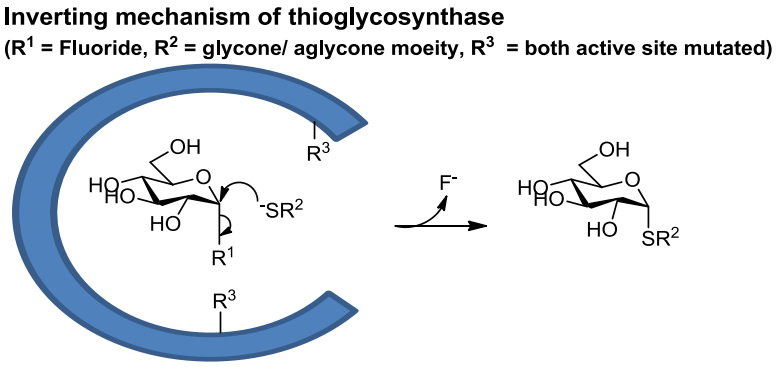


Fig. 1.13: Thioglycosynthase mechanism [67].

1.3.2.5 Benefits and shortcomings of utilising biocatalysis for glycosidic bond formation

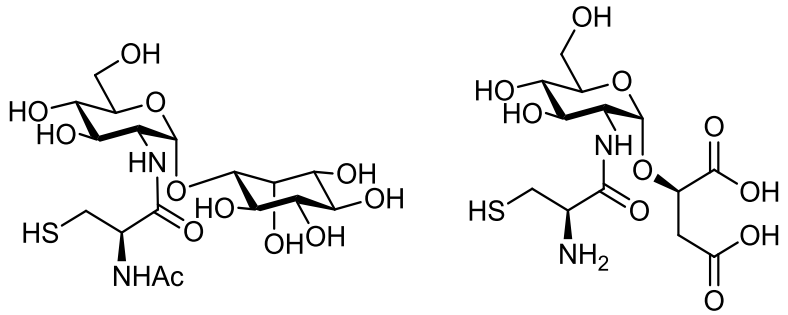
The transformation of glycosidases into either glycosynthases or thioglycoligase has great benefit for the production of glycosides. Reactions catalyzed by these enzymes give good to excellent final product yields without too much difficulty. In addition, mutated glycosidase enzymes are stable, highly soluble and can be used with cheaper and a wider range of donors and acceptors. Nevertheless, there are some challenges associated with converting glycosidases into either glycosynthases or thioglycoligases. Retaining glycosynthases requires an external nucleophile to mimic the role of the mutated nucleophilic residue, that is, the glycosyl donor must be reactivated by an external nucleophile, and this is not always observed. Additionally, enzymes with an inverting mechanism require glycosyl donors that are not available commercially, meaning that they must be chemically synthesized. For thioglycoligases, a major shortcoming is the need for the chemical synthesis of thio-sugars that can act as acceptors; this is often not trivial. Currently, only five retaining α-glycosynthases have been generated, and only two retaining α-thioglycoligases [40], demonstrating the difficulty associated with converting an α-glycosidase into either α-glycosynthases or α-thioglycoligases.

1.4. Project background and rationale

Pathogenic bacteria such as *S. aureus* and *M. tuberculosis* among other bacteria are becoming more resistant to the antibacterial drugs that are used to treat infections caused by them [68]. For example, *S. aureus* has developed several resistant strains including methicillin-resistant *S. aureus* (MRSA) and community-associated MRSA

(CA-MRSA) [68-70], while *M. tuberculosis* has developed resistant strains including multidrug-resistant tuberculosis (MDR-TB), extensively drug resistant tuberculosis (XDR-TB) and total drug resistant tuberculosis [68, 71-75]. This resistance is due to several mechanisms, including the reduction of intracellular drug concentration through excretion via efflux pumps, the modification of the drugs by enzymes in the targeted organism that render them harmless, and the modification of the target site to prevent drug binding [17, 23]. In addition, social factors such as non-compliance of patients to prescribed antimicrobial treatments, poor health service, poor hygiene and poor living conditions also accelerate the process of antibiotic resistance.

The escalation in the numbers of drug-resistant pathogens present a real threat to the global public health, as over a million of people die every year due to these pathogenic bacteria [68]. Such problems call for urgent action, specifically to develop new antibiotics that will be active against these resistant strains. Furthermore, new potential targets that are conserved within the pathogen need to be identified and studied for their potential for the development of new drugs. Currently a specific biosynthetic pathway that is conserved within certain actinomycetes, including the pathogen *M. tuberculosis* and another that is found in *S. aureus* and certain Firmicutes, are being viewed as potential targets for the development of new antibacterial drugs against these organisms [76-79]. These pathways are the mycothiol (MSH) biosynthetic pathway in actinomycetes and the bacillithiol (BSH) biosynthetic pathway in Firmicutes and *S. aureus*. These pathways are essential within the respective organisms for the generation of these essential low molecular weight thiols. MSH and BSH consist of a L-cysteinyl-D-glucosamine moiety linked to an aglycone through an α-glycosidic bond (Figure 1.14). For MSH the aglycone moiety is inositol, while that of BSH is malic acid.



Mycothiol (MSH) **Bacillithiol (BSH)**

Fig. 1.14: The chemical structures of MSH and BSH.

MSH and BSH are biosynthesised in several enzymatic steps that include a glycosyl-transfer, dephosphorylation, deacetylation, acetyl-transferase and ligation reaction with each reaction catalysed by a different enzyme (Figure 1.15) [76, 80-83]. The dephosphorylation and acetyl-transferase reactions only occur in the MSH biosynthetic pathway. MSH is generated through five enzymatic reactions (Figure 1.15A) [76, 80, 81]. The first reaction is catalysed by a glycosyltransferase enzyme known as MshA which transfers *N*-acetylglucosamine from uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) to 1-L-myo-inositol-1-phosphate (1-L-Ins-1-P) to form 3-phospho-1-D-myo-inosityl-2-acetamido-2-deoxy-α-D-glucopyranoside (GlcNAc-Ins-3-P) and UDP. This is followed by the removal of the phosphate group by means of an uncharacterized phosphatase, MshA2, to produce 1-D-myo-inosityl-2-acetamido-2-deoxy-α-D-glucopyranoside (GlcNAc-Ins) and phosphate (P_i). The third reaction involves an *N*-deacetylase enzyme known as MshB which cleaves the acetyl group from the *N*-glucosamine moiety to give glucosamine-inositol (GlcN-Ins) and acetic acid (AcOH). The free amino group of GlcN-Ins is subsequently joined to a cysteine amino acid in the presence of ATP by a ligase enzyme, MshC to yielding cysteine-glucosamine-inositol (Cys-GlcN-Ins) and pyrophosphate (PP_i). The last reaction is the transfer of an acetyl group from acetyl-coenzyme A (AcCoA) to the free amino group of Cys in Cys-GlcN-In as catalysed by MshD, generating MSH and CoASH.

On the other hand, BSH in generated in three enzymatic steps (Figure 1.15B) [82]. The first reaction is catalysed by a glycosyltransferase enzyme known as BshA, which transfers *N*-acetylglucosamine from uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) to L-malate to form *N*-acetylglucosamine-malate (GlcNAc-Mal). This is followed by a reaction catalysed by an *N*-deacetylase enzyme known as BshB which cleaves of the acetyl group from the *N*-glucosamine moiety of GlcNAc-Mal to form glucosamine-malate (GlcN-Mal). The last step is catalysed by BshC, which ligates cysteine (through its carboxyl) to the free amino group of GlcN-Mal to form BSH.

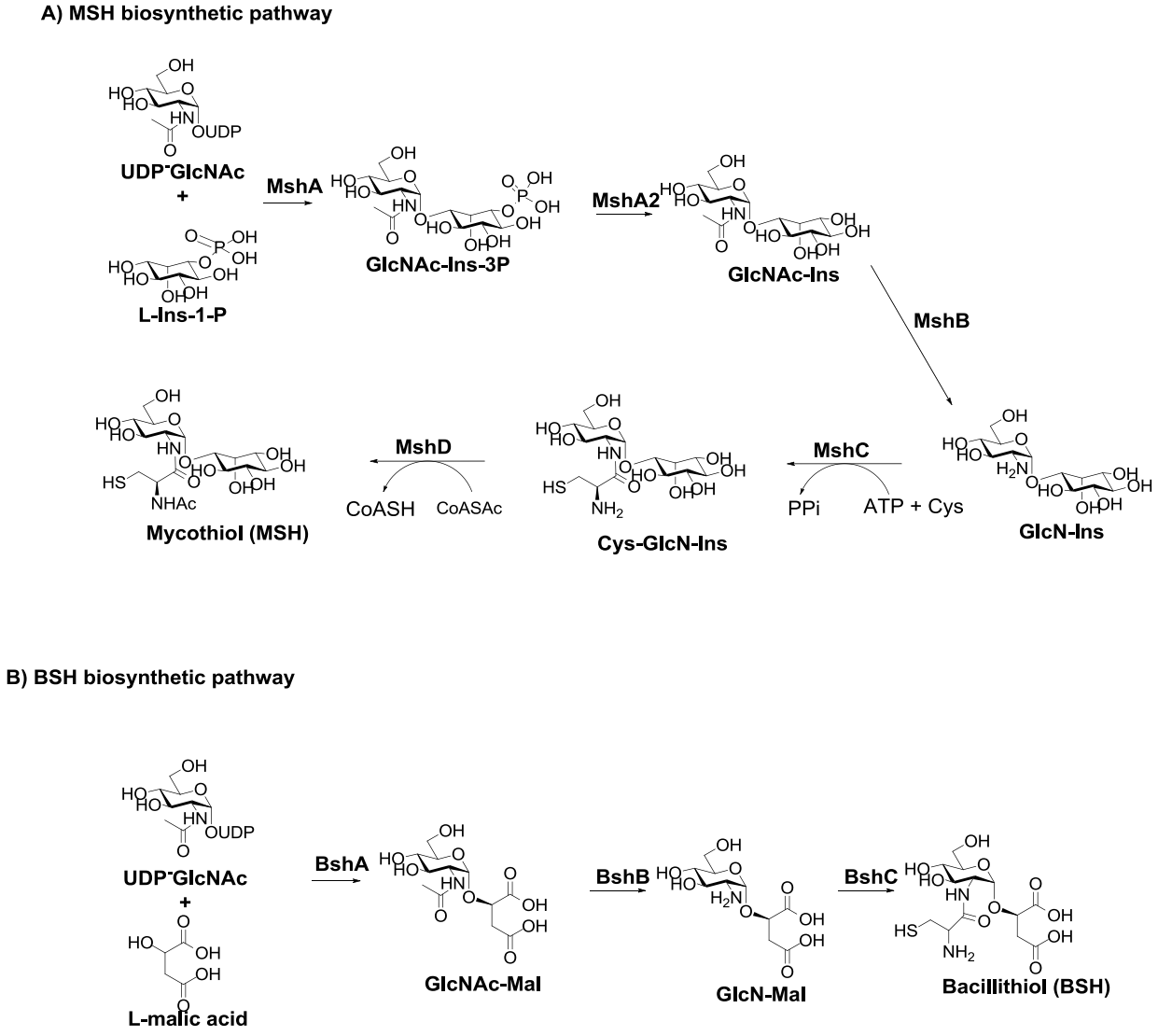


Fig 1.15: The biosynthetic pathways of MSH (**A**) [76] and BSH (**B**) [82], respectively. See text for a detailed description of each step.

These low molecular weight thiols are essential within these microorganisms for several metabolic reactions. They maintain intracellular redox balance in a manner similar to glutathione in eukaryotes, act as co-factors and are used by the microorganisms for the detoxification of xenobiotics, including antimicrobial agents [76, 83-86]. These various processes are facilitated by different MSH and BSH-dependent enzymes, such as mycothiol disulfide reductase (Mtr), mycothiol-*S*-conjugate amidase (Mca), mycothiol-*S*-nitrosoreductase/-formaldehyde dehydrogenase (MscR) in the case of MSH. For BSH, BSH-dependent enzymes include bacillithiol *S*-tranferase (BST) and bacillithiol *S*-conjugate amidase (Bca). Both BSH- and MSH-biosynthetic and -dependent enzymes are being studied to

determine their potential as targets for the development of new antibiotics. However, such studies are hindered by the lack of the crystal structures of some of the enzymes in question, the lack of availability of the substrates of some of the enzymes, resulting in poor characterization of their activities and finally, the lack of appropriate continuous assays that could facilitate the characterisation of these enzymes. In combination, these factors have hampered the development and identification of compounds that specifically inhibit these enzymes and that could be developed into antimicrobial agents.

1.5 Problem identification and previous work

Of the stumbling blocks outlined above that currently hinder the study of MSH- and BSH-related enzymes, the lack of substrate availability is probably one of the biggest to overcome. This is due to the various synthetic difficulties associated with preparing these compounds, one of which is the challenge of generating the α-anomeric glycosidic linkage. In addition, the lack of continuous kinetic enzymatic assays, specifically for the deactylases BshB and MshB that are regarded as targets that has excellent potential for antimicrobial development, also contribute to the slow progress in development inhibitors that target these pathways.

Previous studies in the Strauss group and in the laboratories of our collaborators had identified an α-*N*-acetylglucosaminidase from *Clostridium perfringens* (*C. perfringens*) from family 89 (*Cp*GH89) that has a retaining mechanism as a candidate for the development of an α-glycosynthase and/or α-thioglycoligase catalyst that could be used in the synthesis of MSH and/or BSH biosynthetic intermediates, or of analogues of these compounds. In previous work four potential α-glycosynthases and four potential α-thioglycoligases were generated by mutation of the glycosidase active site at the nucleophile (Glu601) or acid/base (Glu483) residues to serine, alanine, glycine or glutamine [87, 88]. Initial characterisation of the mutants showed no reactivation by external nucleophiles, while the α-thioglycoligases were only evaluated for their hydrolytic activity, but not for their ability to promote thioglycosylation reactions. This project therefore focused on expanding on this initial work by characterisation of the α-retaining thioglycoligases prepared from GH89, and applying these in the synthesis of α-GlcNAc-based

glycosides that could act as MshB and/or BshB substrates or inhibitors. Additionally, a study was launched to develop a general continuous assay that would suitable for activity characterization of these two enzymes.

1.6 Objectives of the project

The main goal of this project was to characterise and apply the α-thioglycoligase enzymes from *Cp*GH89 in the generation α-GlcNAc-based glycosides that can be used as potential alternative substrates and/or inhibitors of MshB and/or BshB. A secondary goal was to develop a continuous assay for the characterization of these enzymes.

Objective 1: Characterisation of the α-thioglycoligases derived from the α-*N*-acetylglucosaminidase *Cp*GH89 for the biocatalytic preparation of α-GlcNAc-based glycosides.

The first objective involved the full characterisation of the α-thioglycoligases generated from *Cp*CH89, including their suitability for use as biocatalysts to prepare α-GlcNAc-based glycosides. This also included testing the α-thioglycoligases for thioglycoligation activity using various glycosyl donors and different thio-molecules as acceptor. Finally, the best enzyme/acceptor/donor combinations were evaluated for their ability to produce α-GlcNAc-based glycosides on preparative scale. The compounds prepared in this way were subsequently tested for biomedical activity. The work pertaining to this objective is described in Chapter 2.

Objective 2: Chemo-enzymatic synthesis and characterization of α-GlcNAc-based glycosides as alternative substrates or inhibitors of MshB and/or BshB.

The best α-thioglycoligase/donor combinations as identified in Objective 1 was subsequently used to generate more α-GlcNAc-based glycosides, specifically with the aim of preparing MshB and/or BshB substrate analogues or inhibitor through biocatalysis. Compounds prepared in this manner were tested as alternative substrate of MshB and BshB. The application of α-thioglycoligases in such work is presented in Chapter 3.

Objective 3: Development of a continuous assay suitable for characterization of the metallodeacetylases, MshB and BshB.

The assays that are currently available for characterisation of MshB and BshB are all discontinuous assays with the exception of one, which has several limitations. The problem with the discontinuous assays are that they use derivatizing reagents, are laborious to execute, and do no lend themselves to high-throughput inhibitor screening. A continuous assay for MshB that makes use of various coupled enzyme reactions was therefore developed. The work executed towards the development of such a coupled-enzyme assay is described in Chapter 4.

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Chapter 2:

The α-thioglycoligase derived from a GH89 α-*N*-acetylglucosaminidase synthesizes α-*N*-acetylglucosamine-based glycosides of biomedical interest

2.1 Advanced Synthesis & Catalysis

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2.1.1 Author's Contribution:

Ndivhuwo Olga Tshililo (NOT) and Andrea Strazzulli (AS) shared first authorship of this manuscript. NOT was responsible for generating various α-*N*-acetyl-thioglycosaminoligases (CpGH89-E483Ala/Ser/Gln); tested their structure-based stability; performed the chemical synthesis of the *O*-glycoside donor α-GlcNAc-DNP, the *S*-glycoside methyl 4-*S*-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-4-thio-α-D-glucopyranoside as standard and the 1-thiol-sugar methyl 4-thio-α-D-glucopyranoside as acceptor. NOT also evaluted the chemical rescue of *Cp*GH89-E483 mutants on small scale with sodium azide as the external nucleophile, as well as testing various thiol molecules as acceptors with the mutants identified as α-*N*-acetyl-thioglycosaminoligases in small scale. Both sets of experiments were done using different donors (2NP/2,4DNP-α-GlcNAc) and was analysed by means of thin layer chromatography (TLC). Lastly, NOT conducted the preparative scale biocatalytic preparation of the α-GlcNAc-based glycosides and "Click" adducts using the best α-*N*-acetyl-thioglycosaminoligase (CpGH89-E483Ala/Ser) and confirmed the structures of the products by NMR and HMRS analysis. The generation of the glycosynthases (*Cp*GH89-E603 mutants) and *Cp*GH89-E483G, chemical rescue of CpGH89-E483 mutants with sodium azide as analyzed by HPAEC-PAD, most of the hydrolytic activity assays, the evaluation of the synthetic efficiency of the mutants, the inhibition study and thermal stability assays were done by the group of Dr. Moracci, with AS doing most of the experiments. The group of Prof. Bedini was responsible for the chemical synthesis of α-GlcNAc-DNP and also assisted with analysis of the NMRs.

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The α-Thioglycoligase Derived from a GH89 α-*N*-Acetylglucosaminidase Synthesises α-*N*-Acetylglucosamine-Based Glycosides of Biomedical Interest

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Abstract: We report here on the preparation of a novel α-thioglycoligase that can be used for the fast and efficient synthesis of α-*N*-acetylglucosamine-based glycosides. Using the α-*N*-acetyl-glucosaminidase from *Clostridium perfringens* of family GH89 (according to the Carbohydrate Active Enzymes classification) as starting point, we prepared mutants in the acid/base residue glutamic acid 483 that were found to have different synthetic efficiencies (maximal yields >80% after 24 h) in the presence of an activated donor and suitable acceptors. The synthetic potential of the Glu483 alanine mutant as an α-thioglycoligase – only the third biocatalyst with this stereospecificity reported to date, and the first selective for the *N*-acetylglucosamine moiety – was demonstrated by producing for the first time *N*-acetyl-α-D-glucosaminyl azide and *N*-acetylglucosamine α-thioglycosides in high yields. To showcase the application of such compounds, we show that they offer the wild-type CpGH89 protection from thermal unfolding, demonstrating their potential as pharmacological chaperones for the treatment of mucopolysaccharidosis IIIB (Sanfilippo syndrome).

Keywords: carbohydrate active enzymes; chemo-enzymatic synthesis; glycoside hydrolases; pharmacological chaperones; reaction mechanism of glycoside hydrolases

Introduction

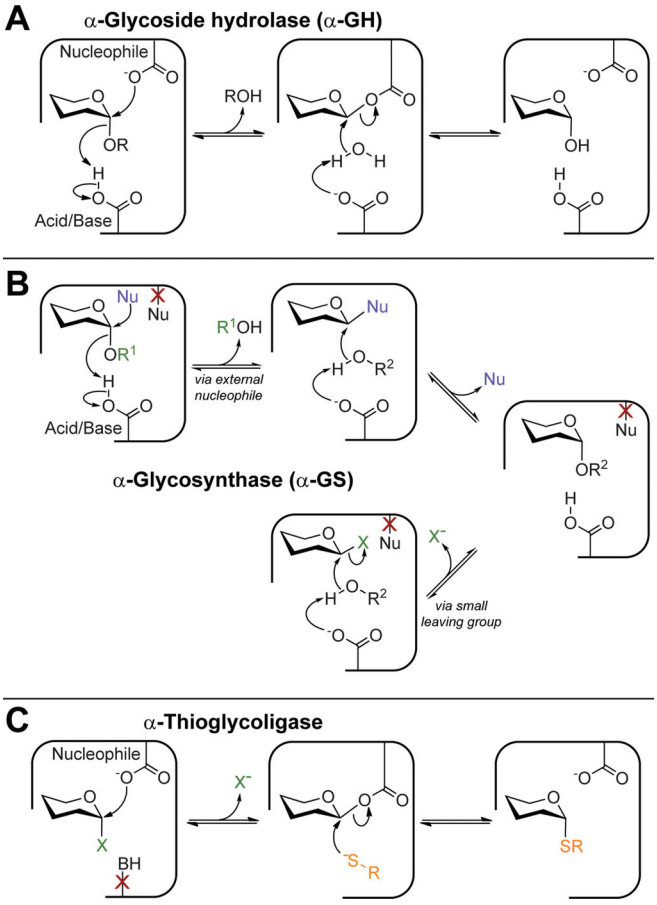
The inhibition of glycoside hydrolases (GHs) is a proven strategy for the study of these interesting enzymes, the discovery of GH-binding molecules, and treatment or study of a variety of metabolic, genetic, and infective diseases.[1] For example, the drugs miglitol and voglibose are used to treat type 2 diabetes based on their competitive inhibition of the α-glucosidase enzyme responsible for the breakdown of starch and smaller oligosaccharides to glucose in the small intestine.[2] However, GH inhibitors can also play a positive role in pharmacological chaperone therapy (PCT) used in lysosomal storage disorders (LSDs) alone or in combination with the enzyme replacement therapy (ERT), helping to maintain the target enzyme in its folded, active form.[3]

The use of GH inhibitors as pharmacological chaperones has become the main treatment option for LSDs where ERT is not possible. A case in point is mucopolysaccharidosis (MPS), a very serious autosomal recessive lysosomal storage disorder caused by the impaired function of enzymes involved in the degradation of heparan sulphate, leading to accumulation

of undegraded glycosaminoglycans in lysosomes. In particular, defective mutations of the human gene encoding for an α-*N*-acetyl-glucosaminidase (NAGLU) causes MPS IIIB, or Sanfilippo syndrome, a tragic rare disease leading to severe neurological disorders.[4] There are no current treatments for MPS IIIB, as gene therapy is in its infancy, and ERT cannot be adopted because the pharmacological enzyme cannot cross the blood–brain barrier to replace the defective enzyme in the nervous system. However, small-molecule chaperones can interact with mutant enzymes to favour their correct conformation and enhance their stability.[5] In this manner pharmacological chaperone therapy (PCT) becomes a viable alternative to ERT in cases where suitable molecules with high affinity for the target enzyme can be identified.

Whether for use as inhibitors or in PCT, the ability of small molecules to selectively bind to their respective targets in the presence of other enzymes with similar substrates would be highly beneficial from a therapeutic perspective. One strategy whereby this can be achieved is by using compounds that have the same anomeric configuration as the natural substrates of the targeted enzymes. However, in the case of α-GHs, this is not easily accomplished. Remarkably, the chemical synthesis of α-linked glycosides of especially *N*-acetyl-glucosamine (GlcNAc) is generally less tractable than that of their β-counterparts. This has severely hampered the discovery and the production in good yields of inhibitors that are selective for α-*N*-acetyl-glucosaminidases, and that can serve as leads for the development of molecules with the potential for use in a clinical setting. Consequently, those compounds that have been shown to inhibit α-*N*-acetyl-glucosaminidase enzymes are in fact repurposed inhibitors of β-*N*-acetyl-glucosaminidases, and do not show selectivity at the anomeric position.[6] Since such a lack of specificity is an undesirable pharmacological feature, the search for α-specific *N*-acetyl-glucosaminidase inhibitors continues. In this regard the discovery of more efficient methods for the reliable synthesis of α-linked glycosides would be extremely useful, and would enable the production of new compounds for testing in such a context.

To address some of the constraints associated with the chemical synthesis of oligosaccharides, such as protection/deprotection strategies and selective activation of the anomeric centre, the use of engineered *retaining* GH enzymes (referring to the anomeric configuration of the product relative to the substrate) was pioneered.[7] This is achieved by mutating either the active site nucleophile or acid/base residues that are required for normal GH catalysis (Scheme 1A) to non-nucleophilic residues, thereby removing the enzymes' ability to hydrolyse glycosidic bonds. To convert the mutant enzymes into biocatalysts, two approaches are followed: in the first, which leads to glycosynthases (GSs), mutation of the active site nucleophile is countered by the use of an activated donor substrate with an appropriate leaving group at the anomeric position (Scheme 1B). If the leaving group is in the same anomeric configuration as the substrate



Scheme 1. ***Mechanisms of α-glycoside hydrolase enzymes and of the biocatalysts that are derived from them.*** **A**: Mechanism of a retaining α-GH showing the active site residues acting as nucleophile and acid/base respectively. R can be a sugar or an alcohol. **B**: Mechanism of an α-glycosynthase (α-GS) derived from the α-GH shown in panel **A** after exchange of its active site nucleophile for a non-nucleophilic residue. Such enzymes can use two types of substrates: if the leaving group is in the α-configuration (–OR^1 in green; with R^1 usually 2-, 4-nitrophenyl, or 2,4-dinitrophenyl), a small external nucleophile (Nu in blue) such as azide or formate is added to allow the reaction with an acceptor (R^2 can be a sugar or an alcohol) to take place, while substrates with small leaving groups (green X = fluoride or azide) in the β-configuration can react directly. In both cases the product is an α-glycoside. **C**: Mechanism of an α-thioglycoligase (α-TGL) derived from the α-GH shown in panel **A** by exchange of its active site acid/base residue for a non-protic/non-nucleophilic residue. In this case the leaving group (X in green) can be 2-, 4-nitrophenyl, 2,4-dinitrophenyl, fluoride or azide, and a thiolate (in orange) is used as acceptor, which acts as nucleophile to form an α-thioglycoside product.

of the wild-type enzyme an external nucleophile such as sodium azide or sodium formate is added to chemically rescue the loss of the active site residue.[8] With some small leaving groups (e.g., fluoride and azide) in a configuration opposite to that of the substrate, chemical rescue is not required (for reviews see ref.[9]). In either case, the product formed accumulates due to the impaired hydrolytic activity of the mutated GH enzyme. In the second approach, which was developed specifically to produce non-hydrolysable thioglycosides, the exchange of the acid/base residue of GHs is again countered by the use of an activated donor with the same anomeric configuration as the substrate but, in this instance, also by an acceptor containing a thiol serving as nucleophile (Scheme 1C). The thioglycoside product accumulates in the reaction, as it is resistant toward enzymatic hydrolysis.[10] Such enzymes are therefore dubbed thioglycoligases (TGL), and their thioglycoside products usually serve as GH inhibitors.

Despite the potency of these approaches – which recently led to an engineered β-hexosaminidase with improved transglycosidase activity[11] – so far α-GSs are limited to α-fucosynthases [from families GH29 and GH95 according to the classification of carbohydrate active enzymes (CAZy, www.cazy.org[12]], α-glucosynthases (GH31), and α-galactosynthases (GH36).[8a,9a,13] In striking contrast, thioglycoligases are even less common. To the best of our knowledge, only two α-TGLs have been produced so far, a thioglucoligase and a thioxyloligase, both belonging to family GH31.[14]

Considering the need for selective inhibitors and molecular chaperones of the medicinally relevant α-*N*-acetyl-glucosaminidase enzymes, we decided to embark on the modification of the catalytic residues of an α-*N*-acetyl-glucosaminidase to produce a novel α-GS and α-TGL able to synthesise α-*N*-acetyl-glucosaminide-containing glycosides. In CAZy, α-*N*-acetyl-glucosaminidases (EC 3.2.1.50) are reported only in family GH89,[12] in which the enzyme from the bacterium *Clostridium perfringens* (CpGH89) has been identified as a useful model system of the human NAGLU. Studies on the 3D-structure of CpGH89 demonstrated that the enzymes belonging to this family followed the classical *retaining* reaction mechanism as opposed to β-hexosaminidases from families GH18, 20, 25, 56, 84, and 85, which hydrolyse substrates containing an *N*-acetyl (acetamido) or *N*-glycolyl group at the 2-position acting as nucleophile and forming an oxazolinium ion intermediate.[15] In addition, mutations giving rise to MPS IIIB were also identified from a homology model of NAGLU.[6,16] We show here that CpGH89 mutants in the nucleophile of the reaction were recalcitrant to act as α-glycosynthases, but that modification of the acid/base with non-nucleophilic amino acids led to efficient α-thioglycoligases capable of producing glycoside analogues when used with suitable acceptors. The potential of such compounds as pharmacological chaperones was subsequently explored by determining the effects of these novel products on parental CpGH89. Taken together, these observations demonstrated that the first α-TGL is capable of efficiently producing α-*N*-acetyl-glucosaminide-containing thioglycosides and *N*-acetyl-α-D-glucosaminyl azide (α-GlcNAc-N_3), a compound that is not commercially available, yet holds promise for the use in bio-orthogonal click chemistry reactions. Such compounds can find broad application in biomedicine for the synthesis of inhibitors and lead compounds of α-GlcNAc-active enzymes.

Results and Discussion

Preparation of α-*N*-Acetyl-glucosaminosynthases

CpGH89 was selected for investigation as potential chassis to produce mutant enzymes able to synthesise *N*-acetyl-α-D-glucosaminide (α-GlcNAc) derived products. The gene encoding for CpGH89 was produced as a truncated version of the wild type, which comprised residues 26–916 as reported previously.[6] The enzyme purified to homogeneity by a single-step purification by immobilised metal affinity chromatography showed the steady state kinetic constants listed in Table 1. Previous studies demonstrated that the enzyme followed a double-displacement retaining mechanism and that the residues glutamic acid 601 (Glu601) and 483 (Glu483) were the nucleophile and the acid/base of the reaction, respectively, with no evidence of the participation in catalysis of the *N*-acetyl group of the substrate (Figure 1A and B).[6] To test if CpGH89 could be converted into an α-GS by following the approach that was demonstrated to be successful for GHs from several families[7] the nucleophile Glu601 was exchanged for non-nucleophilic residues producing the alanine, glycine, and serine mutants (E601A/G/S). These mutant enzymes had no detectable activity on 2-nitrophenyl-*N*-acetyl-α-D-glucosaminide (2NP-α-GlcNAc) and 2,4-dinitrophenyl-*N*-acetyl-α-D-glucosaminide (2,4DNP-α-GlcNAc) as donor substrates. This is in agreement with previous studies on CpGH89[6] and with the 10^3-fold reduction of specific activity observed in other *retaining* glycoside hydrolases mutated in the nucleophile of the reaction.[17] However, remarkably, attempts to chemically rescue the enzymatic activity of the mutants acting on 2- and 2,4DNP-α-GlcNAc at 25 and 37 °C by using high concentrations (up to 1 M) of external nucleophiles (sodium azide, sodium formate, sodium chloride and sodium fluoride) were all unsuccessful and remained so even with the use of excess of enzyme, different reaction temperatures, buffer/pH

Table 1. Steady-state kinetic parameters of wild type CpGH89 and its Glu483 mutants.

CpGH89 mutant (*substrate*)	K_M [μM]		k_{cat} [s^{-1}]		k_{cat}/K_M [$s^{-1}mM^{-1}$]	
2NP-α-GlcNAc	*25 °C*	*37 °C*	*25 °C*	*37 °C*	*25 °C*	*37 °C*
wild type	416 ± 56	190 ± 10	2.31 ± 0.11	23 ± 0.3	5.6	121
E483Q	11 ± 5	10 ± 1	0.033 ± 0.001	0.029 ± 0.001	3.1	2.90
E483A	46 ± 5	50 ± 3	0.076 ± 0.001	0.051 ± 0.001	1.7	1.02
E483S	34 ± 5	62 ± 4	0.064 ± 0.014	0.093 ± 0.002	1.9	1.49
E483G	79 ± 14	68 ± 5	0.022 ± 0.001	0.046 ± 0.001	0.3	0.67
4NP-α-GlcNAc						
wild type	1049 ± 79	1800 ± 300	0.17 ± 0.01	0.35 ± 0.01	0.16	0.19
E483Q	237 ± 19	200 ± 20	0.0062 ± 0.0001	0.0065 ± 0.0001	0.026	0.028
E483A	459 ± 43	800 ± 40	0.0130 ± 0.0004	0.0146 ± 0.0002	0.028	0.017
E483S	443 ± 25	700 ± 70	0.0158 ± 0.0004	0.0240 ± 0.0078	0.036	0.035
E483G	820 ± 220	600 ± 70	0.0027 ± 0.0002	0.0482 ± 0.0163	0.003	0.080
2,4DNP-α-GlcNAc						
wild type	3.2 ± 0.3	6.2 ± 0.6	5.5 ± 0.1	9.9 ± 0.2	1718	1596
E483Q	nd[a]	nd	nd	nd	nd	nd
E483A	nd	nd	nd	nd	nd	nd
E483S	nd	nd	nd	nd	nd	nd
E483G	nd	nd	nd	nd	nd	nd
2,4DNP-α-GlcNAc + 250 mM NaN_3						
E483Q	2.4 ± 0.3	1.3 ± 0.1	1.5 ± 0.1	1.6 ± 0.2	616	1289
E483A	0.7 ± 0.1	1.8 ± 0.3	1.1 ± 0.1	2.3 ± 1.2	1642	1307
E483S	1.1 ± 0.2	1.8 ± 0.2	2.1 ± 0.1	2.4 ± 0.7	2191	1326
E483G	1.7 ± 0.4	3.2 ± 0.4	1.0 ± 0.1	1.9 ± 0.9	582	591

[a] nd = not detectable.

systems, substrate acceptors, and donor/acceptor ratios (data not shown). In addition, no synthesis was observed by using *N*-acetyl-β-D-glucosaminyl azide (β-GlcNAc-N_3) as donor, in an attempt to apply the same strategy that was successful for other α-GSs (Scheme 1B).[8a,9a] We confirmed that this lack of activity was not due to changes in the stability (Figure 2A) or structure (Supporting Information, Figure S1A) of the E601A/G/S mutants. More likely, the shape of the CpGH89 active site prevents entry and/or placement of the acceptor substrates in catalytically relevant positions (Figure 1B). This renders the enzyme resistant to conversion to an α-GS, as has been reported for similar cases.[18] We therefore turned our attention to the conversion of CpGH89 into a novel α-TGL.

Preparation of α-*N*-Acetyl-thioglycosaminoligases

α-TGLs are mutants in which the general acid/base catalytic residue of the enzyme has been replaced by a non-nucleophilic amino acid.[10] The mutant, in the presence of a sugar donor with an excellent leaving group and suitable acceptors with a thiol goup, catalyses the formation of *S*-glycosidic linkages in high yields. The replacement of the general acid/base of CpGH89 with non-acidic residues (E483A/S/Q/G) produced mutants with very low enzymatic activity on 2NP-α-GlcNAc (up to 780-fold less than the wild type), on 4-nitrophenyl-*N*-acetyl-α-D-glucosaminide (4NP-α-GlcNAc, up to 70-fold less) and on 2,4DNP-α-GlcNAc (up to 30-fold less) (Table 1), confirming what was previously reported for the CpGH89–E483A mutant on 4NP-α-GlcNAc as substrate.[6] We confirmed that this lack of activity is not due to structure-based defects introduced by the mutation through circular dichroism (CD) analysis of the protein's secondary structure elements (Supporting Information, Figure S1B) which produced mutants with very low enzymatic activity on 2NP-α-GlcNAc (up to 780-fold less than the wild type), 4-nitrophenyl-*N*-acetyl-α-D-glucosaminide and by comparative analysis of their temperature melting curves (Figure 2B). No significant differences could be observed, indicating that the loss of activity can be directly attributed to the mutation.

Chemical Rescue of the Acid/Base Mutants with NaN_3

To test if the inactivation caused by mutation of the general acid/base residue could be chemically rescued, we used sodium azide (NaN_3) as external nucleophile. In an initial test, the rate of the release of 2,4-dinitrophenolate from the more reactive donor 2,4DNP-α-GlcNAc at 37 °C was measured in the presence of increasing concentrations of sodium azide as catalysed by the Glu483 mutants (Figure 3A). The re-

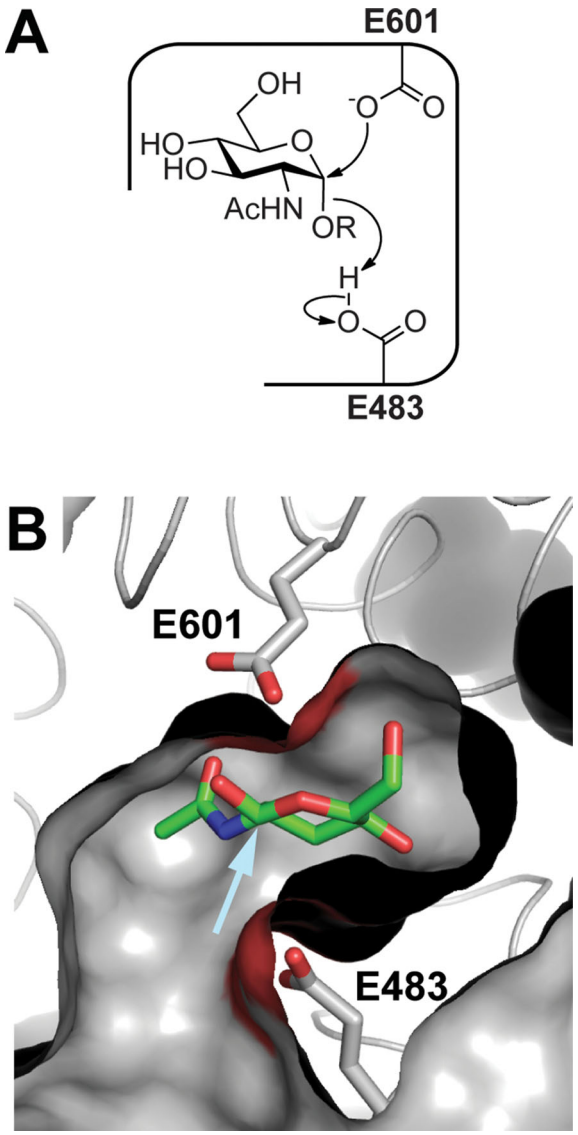


Figure 1. ***CpGH89 catalytic mechanism and active site structure.*** **A**: Mechanism of the initial hydrolytic step catalyced by CpGH89, showing the roles of the active site nucleophile (Glu601) and acid/base (Glu483) residues. **B**: Substrate binding pocket of CpGH89 shown as a section through the solvent-accessible surface with GlcNAc (stick structure with carbon atoms in green) bound. The anomeric carbon that is the point of initial attack by Glu601 (nucleophile) is indicated by an arrow. Both Glu601 and Glu483 are shown as stick structures with carbon atoms in grey.

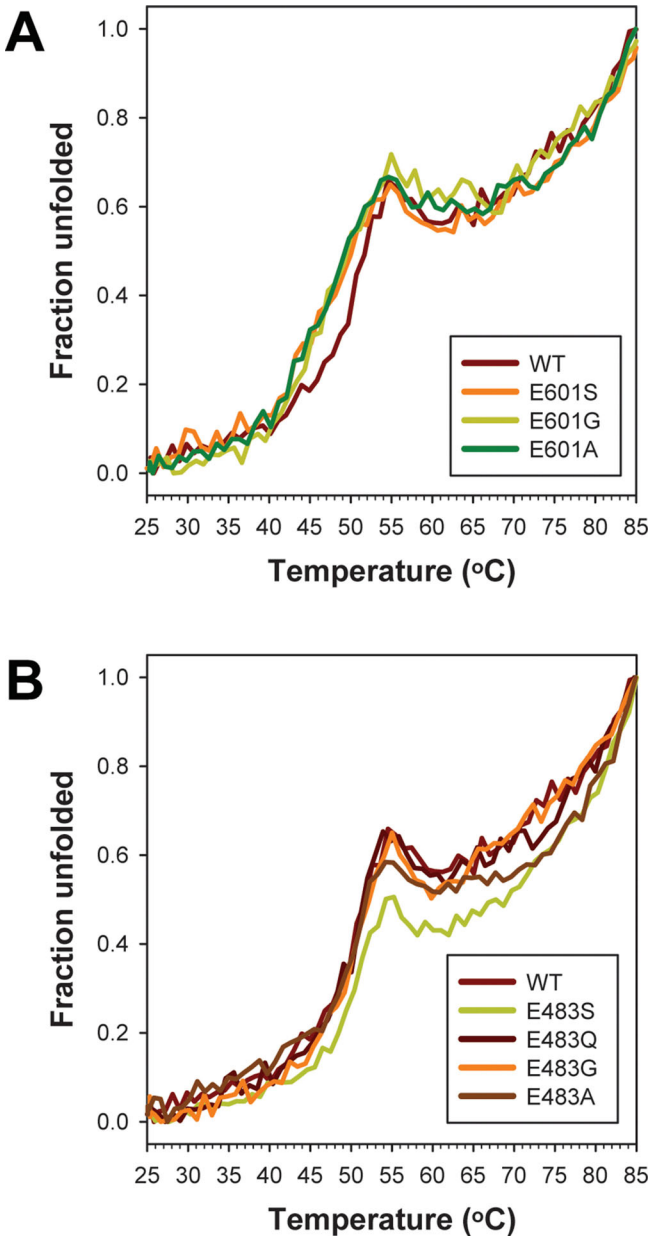


Figure 2. ***CpGH89 structure-based stability.*** **A**: Melting curves of CpGH89 and its Glu601 mutants measured by following the change in CD absorption at 220 nm by heating from 25 °C to 85 °C in intervals of 1 °C each minute. **B**: As for **A**, but for the Glu483 mutants.

sults clearly show that addition of azide restored the mutants' activity, with the CpGH89–E483A and E483S mutants showing the best performance under these conditions. This finding was confirmed by determining the steady-state kinetic constants of the mutants acting on 2,4DNP-α-GlcNAc in the presence of 250 mM sodium azide (Table 1). TLC analysis of these reactions incubated for 16 h showed that the donor was converted to a glycoside with a retention factor (R_f) very similar to that of the control β-GlcNAc-N_3, the closest commercially available analogue of the expected product α-*N*-acetyl-D-glucosamide azide (α-GlcNAc-N_3) (data not shown). These same conditions were subsequently used to determine the synthetic efficiency of all the mutants (calculated as the % products formed from the donor assuming the amount of GlcNAc available in 2,4DNP-α-GlcNAc as 100%) by analysis with high performance anionic exchange chromatography using pulsed amperometric detection (HPAEC-PAD). The efficiency was found to be remarkably high for all the mutants,

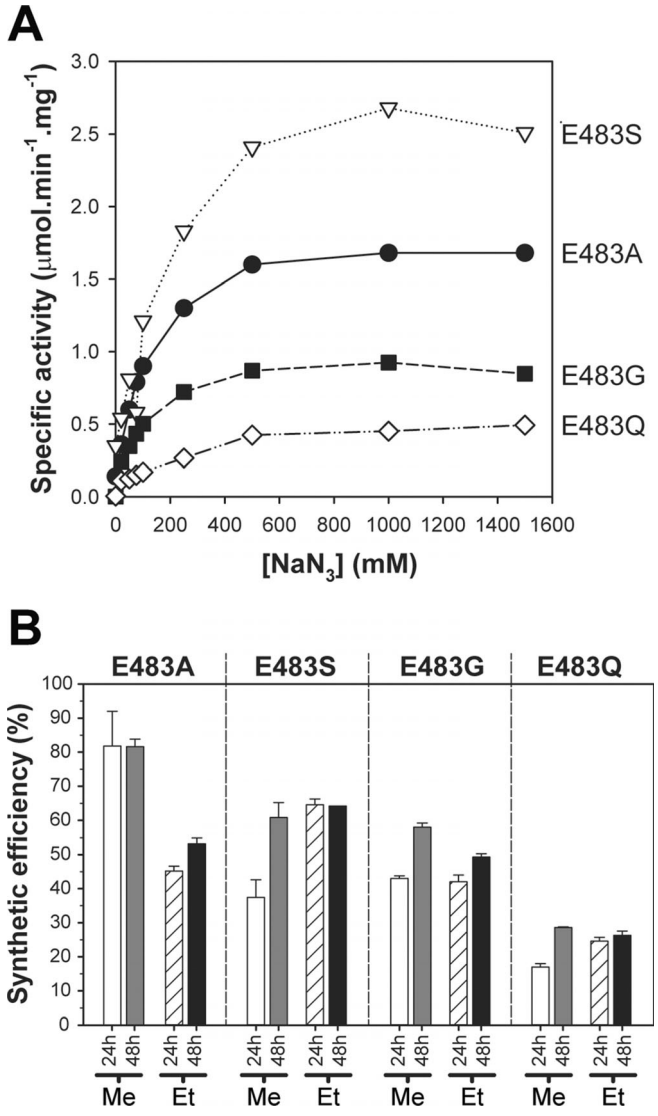


Figure 3. ***Thioglycoligase activity of CpGH89-derived acid/base mutants.*** **A**: Chemical rescue of the activity of the CpGH89–Glu483 mutants in the presence of increasing amounts of sodium azide. The activity was measured at 37 °C using 2,4DNP-α-GlcNAc as donor under standard conditions. **B**: Synthetic efficiency of the CpGH89–Glu483 mutants using 2NP-α-GlcNAc as donor and either methyl thioglycolate (Me) or ethyl thioglycolate (Et) as acceptors. The reaction mixtures were analysed by HPAEC-PAD after 24 and 48 h to quantify the amount of product formed. The synthetic efficiency of the mutants was determined assuming the amount of GlcNAc available in 2NP-α-GlcNAc as 100%. Bars indicate the average value obtained from duplicate experiments; the error bars indicate the standard deviation.

being 70.3±0.1%, 78.6±0.1%, 75.0±1.5% and 81.4±0.8% for E483A/S/Q/G, respectively, while the blank mixture, containing all the reagents but the enzyme, revealed only GlcNAc resulting from the spontaneous hydrolysis of the donor (Supporting Information, Figure S2).

These high yields prompted us to reinvestigate the activity of the mutants at either 25 or 37 °C with all three donors and either low (5 mM) or high (100 mM) concentrations of NaN_3. TLC analysis showed spots corresponding to α-GlcNAc-N_3 for all the mutants but only when 100 mM NaN_3 was used with either 2NP-α-GlcNAc or 2,4DNP-α-GlcNAc as donors; we found that no reaction took place with 4NP-α-GlcNAc (Figure 4). Interestingly, the most productive reactions were found to be those using either 2NP-α-GlcNAc as donor at 37 °C or 2,4DNP-α-GlcNAc at 25 °C. The results also show that when 2NP-α-GlcNAc was used as donor at 37 °C or 2,4DNP-α-GlcNAc at either temperature, hydrolysis (i.e., formation of GlcNAc) was the predominant reaction being catalysed. These findings show that donor reactivity (as mediated by the nature of the leaving group or the reaction temperature) is the primary consideration for activity. However, when reactivity is too high, or in the absence of sufficient acceptor (NaN_3), donor hydrolysis becomes a significant problem. Optimised product formation would therefore entail finding a balance between donor reactivity in the presence of a specific acceptor, and hydrolysis.

Taking these results together, we concluded that the Glu483 mutants were promising candidates for application as α-TGLs and set out to demonstrate this using a range of thiol acceptors.

Evaluating α-TGL Activity using Selected Thiol Acceptors

Encouraged by the chemical rescue of the activity of the CpGH89 acid/base mutants using sodium azide, we decided to perform a comparative evaluation of their α-TGL activity. This was achieved by measuring their synthetic efficiencies when incubated at 37 °C using 2NP-α-GlcNAc as donor (the preferred choice, since it is commercially available and showed high levels of product formation in the reactions with NaN_3). As acceptors, the thiolated esters methyl or ethyl thioglycolate were used, since the pK_a values of their thiol groups (~8.08 at 25 °C for methyl thioglycolate – from the National Center for Biotechnology Information. PubChem Compound Database; CID = 16907, https://pubchem.ncbi.nlm.nih.gov/compound/16907, accessed on August 5, 2016) would ensure that at least half of the molecules in the reaction mixture would be in the more nucleophilic thiolate form at pH 8.0, i.e., the conditions under which the tests were performed. Mutants were incubated using >20-fold molar excess of the donor, and the amount of thioglycoside product formed was measured by HPAEC-PAD analysis of the reaction mixtures after 24 h and 48 h. The yield of converted product in each case was calculated based on the amount of GlcNAc available

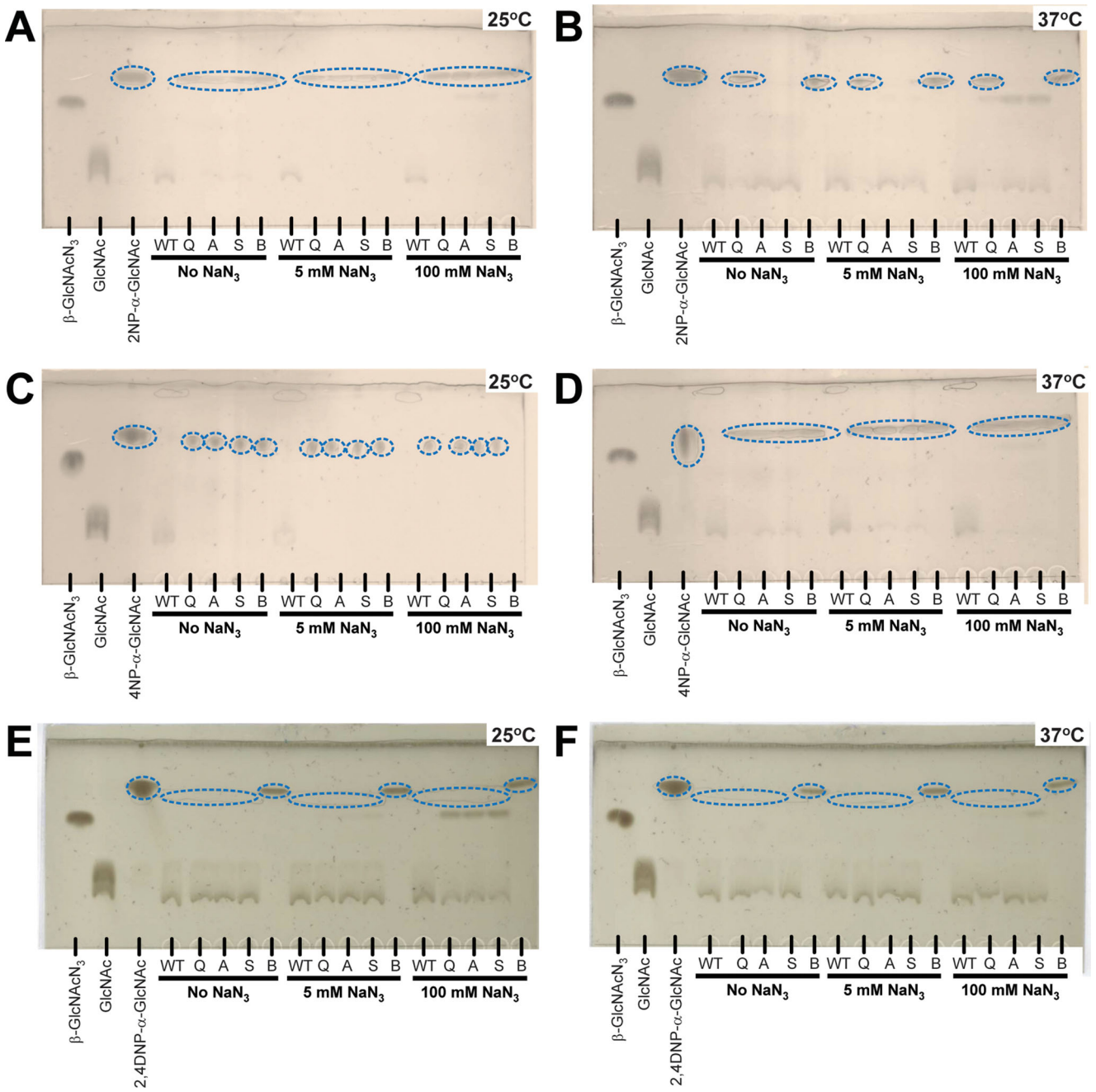


Figure 4. ***Products of the chemical rescue of CpGH89–E483 mutants from different donors as analysed by thin layer chromatography (TLC)***. β-GlcNAc-N_3, GlcNAc, and the respective donor are loaded in the first three lanes of each TLC plate as references. Three sets of reaction mixtures were analysed in each case, with either no NaN_3, or 5 mM or 100 mM NaN_3 present, and with each set containing wild-type enzyme (WT), E483Q (Q), E483A (A), E483S (S) mutant, and a blank reaction without enzyme (B). Reaction mixtures were incubated at either 25 °C or 37 °C, as indicated. Spots that were UV-visible are indicated by dotted circles; other spots were visualised by staining with 10% sulphuric acid in methanol, followed by incubation at 150 °C for 15 min. **A**: Using 2NP-α-GlcNAc as donor at 25 °C. **B**: Using 2NP-α-GlcNAc as donor at 37 °C. **C**: Using 4NP-α-GlcNAc as donor at 25 °C. **D**: Using 4NP-α-GlcNAc as donor at 37 °C. **E**: Using 2,4DNP-α-GlcNAc as donor at 25 °C. **F**: Using 2,4DNP-α-GlcNAc as donor at 37 °C.

in 2NP-α-GlcNAc set as 100%. Blanks showed only unreacted donor (Supporting Information, Figure S3). The results show that in the case of methyl thioglycolate, the CpGH89–E483A mutant is by far the most efficient α-TGL, with a yield of 81.8 ± 10.2% after only 24 h (Figure 3B). With the larger ethyl thioglycolate acceptor the efficiency of the mutants was reduced, and in this case the E483S mutant gave the

best results (64.6 ± 1.70%, also after only 24 h). The E483Q mutant was found to show the least efficient activity, whereas the activity of the E483G mutant corresponded to those of the alanine and serine mutants with ethyl and methyl thioglycolate, respectively.

In the light of these results, we chose the CpGH89–E483A and E483S mutants to investigate their activity as α-TGLs using a variety of thiol acceptors at concentrations of between 2.5 mM and 100 mM. Reactions were performed at 37 °C using 5 mM 2NP-α-GlcNAc as donor in 100 mM sodium phosphate buffer. Based on previous reports that the pK_a of the thiol group is an important consideration in its ability to act as acceptor in a thioglycoligase reaction,[19] we chose the thiols to span a range of pK_a values, and also performed the reactions at various pHs to evaluate if the predominance of the thiolate anion is a prerequisite for the TGL reaction to proceed. Reactions were evaluated by TLC and scored for product formation and hydrolysis as a side-reaction (Table 2). The results show that α-TGL activity is usually observed with thiols that have pK_a values of 8 and lower, i.e., thiols that would be at least 50% ionised at pH 8.0. We found that promoting the ionisation of thiols with higher pK_a values was not practically possible, since the hydrolysis of the donor becomes a significant side reaction at higher pH values. In fact, although the 2NP-α-GlcNAc donor (as an acetal) is expected to show enhanced stability at increased pH, the observed side reaction likely is the result of the higher concentration of HO^- by acting as nucleophile on the enzyme-GlcNAc intermediate. Indeed, we observed that for the thiosugar methyl 4-deoxy-4-thioglucoside and for cyclohexanethiol, both of which have pK_a values of 9.5 and above, GlcNAc was the major product formed. These findings indicated that the CpGH89–E483A and E483S mutants have excellent α-TGL activity, but only if the thiol acceptor is carefully chosen.

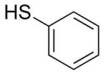
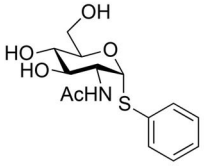
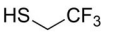
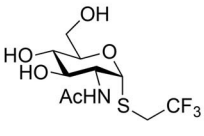
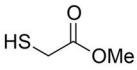
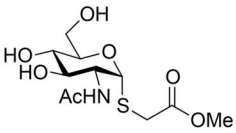
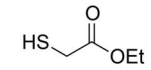
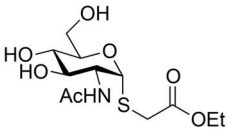
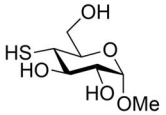
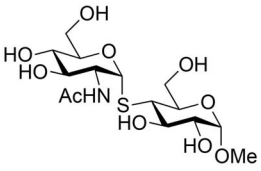
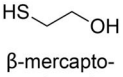
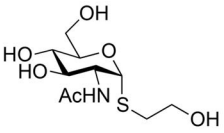
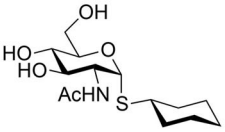
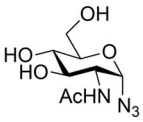
CpGH89–E483A/S Mutants as Preparative Scale α-TGL Biocatalysts

The excellent synthetic efficiency of the CpGH89–E483A mutant motivated us to determine if its α-TGL activity would also translate to reactions on a preparative (multimilligram) scale, and allow the synthesis and purification of α-GlcNAc-N_3 (using NaN_3 as external nucleophile) or α-GlcNAc thioglycosides (using thiol acceptors with pK_a values of 8 or lower). Towards this end, we performed biocatalysis reactions using CpGH89–E483A and/or E483S mutants using ~0.08 mmol 2NP-α-GlcNAc as donor and a 20-fold molar excess of acceptor at 37 °C. Reaction progress was monitored by TLC, and once being judged as complete, the reaction product was purified by means of four sequential steps that removed the protein, buffer and nitrophenol side-product, respectively, before the final product was purified by flash chromatography on silica. The purified products were analysed by ^1H and ^{13}C NMR and HR-MS analysis to confirm their structures, including the expected α-configuration of the newly-formed glycosidic bond. The preparative scale yields were also determined based on the amount of donor used. These values, summarised in Table 2, show that the small scale synthetic efficiencies were excellent predictors of the mutants preparative scale abilities, with the CpGH89–E483A mutant also giving preparative yields in excess of 80%. The lower synthetic yields observed in some cases are due to less favourable transglycosylation/hydrolysis ratios (compare the columns showing donor hydrolysis and product formation for each enzyme and substrate in Table 2) and, in some cases to difficulties encountered in the purification of the products. However, it is worth mentioning that the overall efficiency of the chemo-enzymatic approach shown here is much better than the classical chemical synthesis strategy that needs many more steps. Moreover, the yields obtained for the compounds prepared in this study are comparable to the overall yield of 35% reported for one of these compounds (entry 1, Table 2) prepared in six steps by traditional synthetic methods.[21] These findings confirm that the CpGH89–E483A mutant is an excellent α-TGL biocatalyst. In addition, this mutant also provides a means for the preparation of α-GlcNAc-N_3, a compound that is currently not commercially available and, according to the chemical database SciFinder, has never been described before. Nevertheless, it yet has multiple potential uses – including as azide substrate in the exceedingly versatile "click" reactions such as copper(I)-catalysed- and strain-promoted azide–alkyne cycloadditions (CuAAC and SPAAC).[22] Taken together, the CpGH89–E483A mutant opens routes to the facile preparation of a variety of α-linked *S*-glycosides and triazoles of high potential value that were previously not synthetically tractable.

Effect of the α-TGL Products on CpGH89

Since GH enzymes cannot hydrolyse the *S*-glycosidic bond, we next sought to demonstrate the potential of the biocatalytically-prepared GlcNAc α-thioglycosides as inhibitors of *N*-acyl-glucosaminidases. This was done by evaluating the ability of the compounds to bind to and inhibit the GH activity of wild-type CpGH89. We included in these tests commercially obtained β-GlcNAc-N_3 to demonstrate the importance of the configuration of the glycosidic bond, as well as GlcNAc-α(1→4)-(methyl 4-deoxy-4-thioglucoside) [GlcNAc-*S*-(Me4-thioGlc)], the expected product of

Table 2. The α-thioglycoligase activity of CpGH89–E483A and E483S with various thiol acceptors or sodium azide, and 2NP-α-GlcNAc as donor.

Entry	CpGH89 mutant	Acceptor	pK_a[a]	Expected product	Donor hydrolysis[b]	Product formation[c]	Preparative scale yield [%][d]
1	E483A	 thiophenol	6.6		pH 6.5 + pH 7.3 + pH 8.0 +	pH 6.5 ++ pH 7.3 ++ pH 8.0 ++	18
2	E483S				pH 6.5 + pH 7.3 + pH 8.0 +	pH 6.5 +++ pH 7.3 +++ pH 8.0 +++	
3	E483A	 2,2,2-trifluoro-ethanethiol	7.6		pH 7.3 + pH 8.0 +	pH 7.3 + pH 8.0 +	15
4	E483S				pH 7.3 + pH 8.0 +	pH 7.3 ++ pH 8.0 ++	
5	E483A	 methyl thioglycolate	8.1		pH 7.3 + pH 8.0 +	pH 7.3 +++ pH 8.0 +++	83
6	E483S				pH 7.3 + pH 8.0 +	pH 7.3 +++ pH 8.0 +++	49
7	E483A	 ethyl thioglycolate	8.1		pH 7.3 + pH 8.0 +	pH 7.3 +++ pH 8.0 +++	46
8	E483S				pH 7.3 + pH 8.0 +	pH 7.3 +++ pH 8.0 +++	33
9	E483A	 methyl 4-deoxy-4-thioglucoside	9.5		pH 7.3 + pH 8.0 ++	pH 7.3 - pH 8.0 -	
10	E483S				pH 7.3 + pH 8.0 ++	pH 7.3 - pH 8.0 -	
11	E483A	 β-mercapto-ethanol	9.7		pH 7.3 ++ pH 8.0 ++	pH 7.3 - pH 8.0 +	
12	E483S				pH 7.3 + pH 8.0 +	pH 7.3 - pH 8.0 +	
13	E483A	 cyclohexanethiol	10.7		pH 7.3 ++ pH 8.0 ++	pH 7.3 - pH 8.0 -	
14	E483S				pH 7.3 + pH 8.0 +	pH 7.3 - pH 8.0 -	
15	E483A	NaN_3 sodium azide					79

[a] Values taken from the National Center for Biotechnology Information (NCBI) PubChem Compound Database (https://pubchem.ncbi.nlm.nih.gov/), or from ref.[20] The pK_a value for methyl 4-deoxy-4-thioglucoside was calculated using ChemAxon's pK_a predictor.

[b] Relative extent of donor hydrolysis based on the intensity of the spots representing GlcNAc and 2NP-α-GlcNAc as visualised from the TLC analyses of the reaction mixtures.

[c] Relative extent of product formation based on the intensity of the anticipated product spot (identified by comparison with a standard, if available) as visualised from the TLC analyses of the reaction mixtures.

[d] Yield of purified product prepared from preparative scale biocatalytic transformations, based on the amount of 2NP-α-GlcNAc donor used as starting material.

the TGL reaction with methyl 4-deoxy-4-thioglucoside as acceptor. While we were not able to prepare this compound through biocatalysis, we prepared it by chemical synthesis to act as standard for the TGL evaluation tests, and it was therefore available for testing.

To quantify the inhibitory effect we measured the specific activity at standard conditions of wild-type CpGH89 at 600 μM of each compound. This concentration was chosen based on this amount of *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate (PUGNAc), a known competitive inhibitor of CpGH89.[6] leading to 20% residual activity. Among the α-TGL products tested, the products of the reactions with methyl and ethyl thioglycolate (GlcNAc-*S*-CH_2CO_2Me and GlcNAc-*S*-CH_2CO_2Et, respectively) showed the highest inhibition with 79% and 62% of residual activity, respectively (Figure 5A).

In order to evaluate if the products obtained by chemical rescue and by the TGL approach could protect wild-type CpGH89 from unfolding by binding to the enzyme, we performed differential scanning fluorimetry (DSF) experiments in the 25–94 °C temperature range and in the presence of 10 mM of each product, the highest concentration used for allosteric pharmaceutical chaperones (PCs).[23] As positive controls we used PUGNAc (0.2 mM) and two other known *N*-acetylglucosaminidase inhibitors: 2-acetamido-1,2-dideoxynojirimycin (2AcDNJ, 0.1 μM) and 6-acetamido-6-deoxycastanospermine (6AcCAS, 2.8 μM), i.e., at concentrations 30-fold higher than their respective K_i or K_d values.[6] The thermal stability analysis (Figure 5B) revealed that the three inhibitors 6AcCAS, PUGNAc and 2AcDNJ increased the melting temperature (T_m) of wild-type CpGH89 by 6.21 °C, 3.76 °C, and 2.11 °C, respectively (Figure 5C). Among the products of the α-TGL, the best stabilising effect was produced by α-GlcNAc-N_3 (ΔT_m = 4.02 °C), while β-GlcNAc-N_3 did not show any stabilisation of the wild-type CpGH89. This finding demonstrates the importance of the configuration of the glycosidic bond for binding, and as a potential factor in

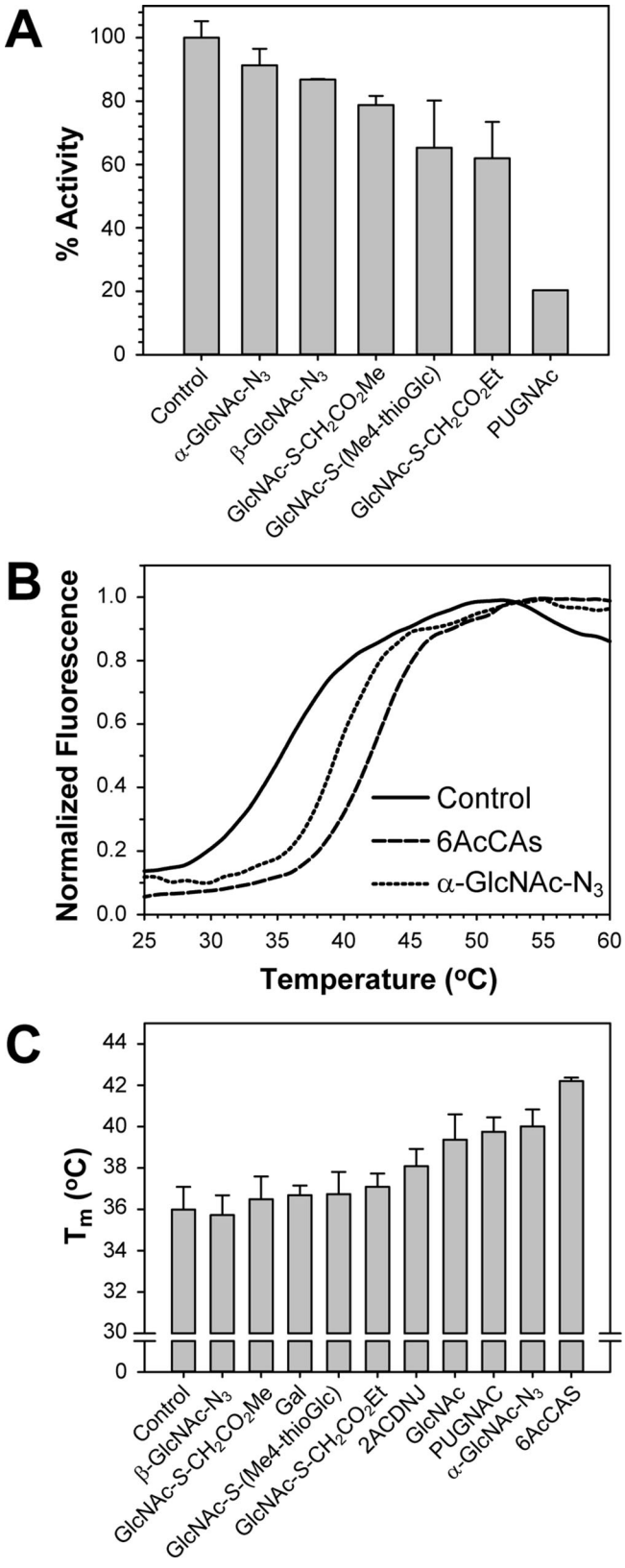


Figure 5. ***Effect of the α-thioglycoligase products on CpGH89 activity and stability.*** **A**: Effect by known inhibitors and α-TGL products on the activity of wild type CpGH89. Bars indicate the average value obtained from duplicate experiments; the error bars indicate the standard deviation. **B**: Denaturation curves followed by differential scanning fluorimetry (DSF) of wild type CpGH89 in the absence of ligands (control) and in the presence of the 6AcCAS inhibitor and α-GlcNAc-N_3 are shown as case examples. **C**: The increasing melting temperatures (T_m) of wild type CpGH89 in the presence of the indicated ligands as determined by DSF. Bars indicate the average value obtained from triplicate experiments; the error bars indicate the standard deviation.

establishing selectivity. GlcNAc, the product of the hydrolysis reaction, also significantly improved the thermal stability of the enzyme ($\Delta T_m = +3.38\,°C$) compared to galactose ($\Delta T_m = +0.70\,°C$), indicating that the stabilising effect of a carbohydrate-derivative molecule could be directly related to the donor −1 subsite.[16] It is worth noting that α-GlcNAc-N_3, which is the second-best PC after 6AcCAS, showed no inhibition of CpGH89 at 0.6 mM concentration (Figure 5A), a characteristic highly recommended in second generation PCs as they can promote the correct folding of the defective target enzyme with no detrimental effect on its activity in the lysosome.[3,24] In addition, as already stated above, α-GlcNAc-N_3 can be used as azide substrate in "click" reactions, thus, showing interesting potential as lead for novel α-linked PC molecules to test in pharmacological chaperone therapies for MPS IIIB.

Conclusions

Stable α-linked analogues of GlcNAc glycosides hold great potential for the development of new selective therapies for a range of diseases, for example, compounds that can act as inhibitors of the enzymes involved in the biosynthesis of essential cofactors in pathogenic organisms,.[25] or by stabilising the structures of defective mutant variants of key metabolic enzymes whose deficiency lead to genetic diseases.[4,5] However, such compounds are not easily obtained through chemical means due to the requirement for multistep synthesis with several protection/deprotection protocols. Here we have demonstrated that α-GlcNAc-N_3 and several α-GlcNAc thioglycosides can be obtained by preparative scale biocatalysis using an engineered mutant of the α-*N*-acetyl-glucosaminidase from *Clostridium perfringens* (CpGH89) in which its catalytically essential acid/base residue (Glu483) has been replaced by alanine. The development of this α-*N*-acetyl-thioglycosaminoligase lowers the barrier to the access of α-linked GlcNAc glycoside analogues significantly, and should facilitate the investigation of such compounds as therapeutic agents. In this study we highlight the potential scope for such compounds by showing their ability to inhibit or stabilise the wild-type form of CpGH89, a proxy for the NAGLU enzyme whose deficiency causes mucopolysaccharidosis IIIB, or Sanfilippo syndrome.

Experimental Section

General Materials and Methods

The donor substrates 2- and 4-NP-α-GlcNAc were obtained from Carbosynth (Compton, U.K.) while 2,4DNP-α-GlcNAc was prepared by chemical synthesis using a previously published protocol.[26] Methyl 4-deoxy-4-thioglucoside and GlcNAc-*S*-(Me4-thioGlc) were prepared by chemical synthesis as outlined in the Supporting Information. General chemicals, reagents, and media were purchased from Sigma–Aldrich or Merck Chemicals (Darmstadt, Germany) and were of the highest purity. Solvents used for reactions were Chromasolv HPLC grade solvents (Sigma–Aldrich), and the ethyl acetate (EtOAc), methanol (MeOH) and water used for purification were purchased from Merck Chemicals. Protein purifications were conducted on an Äkta Prime system (GE Healthcare). Spectrophotometric assays were performed on a Cary 100 Scan spectrophotometer (Varian, Australia), coupled with a thermally controlled Peltier system. High performance anionic exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was performed on a Thermofisher Scientific system equipped with a CarboPac PA200 Analytical (2×205 mm) column. Solid phase extraction (SPE) columns (Phenomenex Strata®) were obtained from Separations (Cape Town, South Africa).

Cloning and Mutagenesis of CpGH89 Wild Type and Mutants

The synthetic *CpGH89* wild type and E601A mutant genes were custom-made by GeneArt Gene Synthesis (Thermofisher Scientific) in pET28a vector, with N-terminal 6×His tag. The mutants E601G/S and E483G were prepared from the pET28a-CpGH89wt by using the GeneTailor Site-Directed Mutagenesis system (Invitrogen) with the following synthetic oligonucleotides (PRIMM, Milan, Italy).

E601G_fwd: 5′-TATTGGTATTACACCGGGCGCCATTAATACCAATCCGC-3′
E601G_rev: 5′-GCGGATTGGTATTAATGGCGCCCGGTGTAATACCAATA-3′
E601S_fwd: 5′-TGGGTATTGGTATTACACCGTCAGCCATTAATAC-3′
E601S_rev: 5′-CGGTGTAATACCAATACCCACCATATGTTC-3′
E483G_fwd: 5′-ATGGTGTGGATCCGTTTCATGGTGGTGGTAATACCGGTGATCT-3′
E483G_rev: 5′-AGATCACCGGTATTACCACCACCATGAAACGGATCCACACCAT-3′
E483A_fwd: 5′-GGATCCGTTTCATGCAGGTGGTAATACCGG-3′
E483A_rev: 5′-CCGGTATTACCACCTGCATGAAACGGATCC-3′
E483S_fwd: 5′-GGATCCGTTTCATTCAGGTGGTAATACCGG-3′
E483S_rev: 5′-CCGGTATTACCACCTGAATGAAACGGATCC-3′
E483Q_fwd: 5′-GGATCCGTTTCATCAAGGTGGTAATACCGG-3′
E483Q _rev: 5′-CCGGTATTACCACCTTGATGAAACGGATCC-3′

PCR-generated constructs were verified by sequencing.

Expression and Purification of CpGH89 Wild Type and Its mutants

CpGH89 wild type and mutants were expressed in *E. coli* strain JM109 (DE3) or BL21*(DE3). The cells transformed with the appropriate construct were grown at 37 °C in 2 L of Luria-Bertani (LB) broth supplemented with kanamycin (50 μg.mL^{-1}). Gene expression was induced by the addition of 1 mM IPTG when the culture reached an OD_{600} of 1.0. Growth was allowed to proceed for 16 h, and cells were harvested by centrifugation at 5000 *g*. The resulting cell pellet

was resuspended in 50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl and 1% TRITON-X100 with a ratio of 5 mL g^{-1} cells, followed by incubation at 37 °C for 1 h with 20 mg of lysozyme (Fluka) and 25 U g^{-1} cell of DNAseI (SIGMA). Cells were lysed by 5 sonication cycles and cell debris was removed by centrifugation at 12000 *g* for 30 min. The cell free extract (CFE) was loaded on a HisTrap FF column (GE-Healthcare) equilibrated with 50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl (Buffer A). After an initial wash-step (20 column volumes) with Buffer A, the protein was eluted at 250 mM imidazole in Buffer A. The active fractions were pooled, dialysed against 20 mM sodium phosphate buffer, pH 7.3, 150 mM NaCl (PBS buffer) and stored at 4 °C. The protein concentration was determined with the Bradford assay (Bio-Rad). After this procedure the enzymes were more than 95% pure by SDS-PAGE, with an approximate final yield of 20 mg L^{-1} of culture.

Comparative Structural Analysis

CD spectroscopy and thermal analysis were conducted using a Photophysics Chirascan-plus spectrometer at 25 °C. The samples contained 0.5 mg mL^{-1} protein in 100 mM sodium phosphate buffer (pH 7.3). The optimum wavelength for analysis of thermal stability was determined by a CD absorption scan from 200–300 nm at 25 °C. The thermal stability experiments were conducted by heating the samples from 25 °C to 100 °C and measuring the changes in CD absorption at 220 nm.

CpGH89 Hydrolytic Activity Assay

Hydrolytic activity assays for CpGH89 wild type and mutants at 25 °C were conducted using a microplate-based continuous spectrophotometric assay measuring the release of the nitrophenolate from the respective donor that was used. For assays conducted at 25 °C, reactions were performed in triplicate in a final reaction volume of 250 µL. The assays contained 2 µM enzyme, 100 mM sodium phosphate buffer (pH 7.3), and substrate concentrations in a range varying between 0–2.5 mM. The liberation of the nitrophenolate ion was monitored at 400 nm ($\varepsilon = 1.8$ $mM^{-1}cm^{-1}$ and 13.3 $mM^{-1}cm^{-1}$ for 2- and 4-nitrophenolate, respectively) for 10 min. Assays conducted at 37 °C were performed in a final reaction volume of 1 mL. The assays typically contained 220 nM of wild type and 1.3–4.11 µM of mutant enzymes, in 100 mM sodium phosphate buffer (pH 7.3), and substrate concentrations in a range varying between 0.5 µM and 5 mM. The liberation of the nitrophenolate ion was monitored at 405 nm ($\varepsilon = 3.63$ $mM^{-1}cm^{-1}$, 16.39 $mM^{-1}cm^{-1}$and 11.92 $mM^{-1}cm^{-1}$ for 2-, 4- and 2,4-dinitrophenol, respectively) for 10 min. The rates of product formation were calculated using linear regression analysis of the initial linear portions of the progress curves; these were plotted against the substrate concentration to obtain the activity profiles. The kinetic parameters k_{cat} and K_M were obtained by performing a least squares fitting of the data to the Michealis–Menten equation. One unit of enzyme activity was defined as the amount of enzyme catalysing the hydrolysis of 1 µmol of substrate in 1 min under the conditions described. Blank mixtures, containing all the reagents but the enzyme(s) were incubated at the same conditions and used to subtract the spontaneous hydrolysis of the donor(s).

Chemical Rescue with Sodium Azide

The chemically rescued hydrolase activity of the four CpGH89 acid/base mutants (E483A/S/Q/G) was measured in 100 mM sodium phosphate buffer at pH 7.3 using 5 mM 2,4DNP-α-GlcNAc and increasing the concentrations of sodium azide (between 50 mM and 1.5 M) at 37 °C. The specific activity was measured as described for the hydrolytic activity assay above. Blank mixtures, containing all the reagents but the enzyme(s) were incubated at the same conditions and used to subtract the spontaneous hydrolysis of the donor(s). Aliquots of the reaction mixtures (20 µL) incubated for 16 h were analysed by thin layer chromatography (TLC) as described previously.[27] Briefly, reaction mixtures were loaded on a silica gel 60 F_{254} plate by using EtOAc/MeOH/H_2O (70:20:10 v/v) as eluent and were detected by exposure to 10% sulphuric acid in methanol, followed by charring at 170 °C. The efficiency of each mutant was evaluated by HPAEC-PAD analysis at 35 °C using a flow rate 0.4 mL min^{-1} and 16 mM sodium hydroxide (NaOH) as eluent. For quantification, a calibration curve was created with three different amounts (0.25, 0.75 and 1.5 nmol) of the α-GlcNAc-N_3, and cellobiose (0.75 nmol) was included as internal standard. The synthetic efficiency of the mutants was determined assuming the amount of GlcNAc available in 2,4DNP-α-GlcNAc as 100%. All the samples were analysed in duplicate.

Evaluating the α-Thioglycoligase Activity of CpGH89–E483A/S with Selected Thiols

The α-thioglycoligase activities of the CpGH89–E483A/S mutants were evaluated using 2NP-α-GlcNAc as the donor. All thioglycoligase reactions were performed using 100 mM sodium phosphate buffer, with pH ranging from 5.8–8.0 (values indicated in Table 2), 1.33 µM enzyme and 5 mM 2NP-α-GlcNAc in a final volume of 200 µL. The final thiol concentration ranged from 2.5 mM to 100 mM. The reactions were incubated at 37 °C, and the mixtures were analysed by TLC with ethyl acetate/methanol/water (EtOAc/MeOH/H_2O) (70:20:10 v/v) using UV light to detect the nitrophenol group, and staining with 10% sulphuric acid in methanol, followed by incubation at 150 °C for 15 min, to visualise all other components. For each reaction 4 × 5 µL of the solution were spotted on the TLC.

Synthetic Efficiency of CpGH89–E483 Mutants with Methyl and Ethyl Thioglycolate as Acceptor

To determine the synthetic efficiency of the four CpGH89 acid/base mutants (CpGH89–E483A/S/Q/G), reactions were performed at 37 °C using 100 mM sodium phosphate buffer (pH 8.0), 1.33 µM enzyme, 5 mM 2NP-α-GlcNAc as donor and 106 mM methyl or ethyl thioglycolate as acceptor (supplied with 17.9 mM DTT) in a final volume of 200 µL. At time intervals (0–48 h) aliquots (10 µL) were withdrawn, diluted 1:100 with water and analysed by HPAEC-PAD at 35 °C using a flow rate of 0.4 mL. min^{-1} and the following elution program: [segment 1] 0–12′ isocratic 16 mM NaOH, [segment 2] 12–42′ up to 96 mM NaOH, [segment 3] 42–52′

step to 160 mM NaOH. The total amount of the expected thioglycoside product was determined by using a calibration curve with three different amounts (0.53, 1.06 and 2.12 nmol of α-GlcNAc-*S*-CH_2CO_2Me and 0.25, 0.75 and 1.5 nmol of α-GlcNAc-*S*-CH_2CO_2Et, respectively) of the product, and cellobiose (0.73 nmol) as internal standard. The authentic compounds used to obtain the calibration curves were obtained by preparative scale biocatalysis as outlined below. The synthetic efficiency of the mutants was determined assuming the amount of GlcNAc available in 2NP-α-GlcNAc as 100%. All the samples were analysed in duplicate.

Preparative Scale Biocatalysis

Preparative scale α-TGL reactions were carried out in 15 mL of 100 mM sodium phosphate buffer (pH 8.0). 2NP-α-GlcNAc (0.026 g, 0.076 mmol) was added as the donor, followed by addition of thiol (20 equiv.) or 100 mM sodium azide as the acceptor. DTT (0.160 mM, 0.370 mg, 0.0024 mmol,) was added as reducing agent to prevent disulphide bond formation in the case of the thiol acceptors. The reaction was initiated by addition of the thioglycoligase catalyst (1.33 μM of either CpGH89–E483A or CpGH89–E483S) to the reaction mixture followed by incubation at 37 °C. Reaction progress was monitored and analysed by TLC (EtOAc/MeOH/H_2O; 70:20:10 v/v). Upon completion (usually after overnight incubation), the reaction was filtered through a syringe filter (13 mm GHP Acrodisc® filter with 0.2 μM GHP membrane) to remove the protein. The filtered mixture was loaded onto an SPE column (Phenomenex Strata C18-U, 55 μm, 70 A, 10 g/60 mL) and the crude product was eluted with methanol. The fractions with the crude product were pooled together and concentrated by rotary evaporation. In the case of reactions with acceptors that do not proceed close to completion (as in the case of thiophenol), the crude product was treated with wild-type CpGH89 for 72 h under standard conditions to hydrolyse any residual donor that may be present. The enzyme was subsequently removed as described above. The crude product was purified by flash column chromatography on silica (EtOAc/MeOH/H_2O; 70:20:10 v/v). Any residual nitrophenol was removed by passing the purified product through Phenomenex Strata® Phenyl (55μm 70 Å) SPE column, eluting with water. The final purified products were obtained following by freeze drying for three days to remove any water, and were fully characterised by ^{1}H NMR, ^{13}C NMR and MS-ESI or HR-MS (see the Supporting Information for data).

CpGH98 Wild Type Inhibition assay and Thermal Stability

The inhibitory effects of the different compounds (0.6 mM each) were assayed by measuring the specific activity of CpGH89 wild type on 2NP-α-GlcNAc in 100 mM phosphate buffer (pH 7.3) at 37 °C as reported above. Thermal stability of CpGH89 wild type was measured by DSF as previously reported[23] with some modifications. Briefly, CpGH89 wild type (2.5 μg) was incubated in citrate phosphate buffer and SYPRO Orange dye 5X, in a total volume of 25 μL in the presence of the different compounds under test. Samples were heated from 25 to 94 °C in a Real-Time Light Cycler (Bio-Rad, Milan, Italy). Thermal stability scans were performed at 0.2 °C min^{-1} and the SYPRO Orange fluorescence was normalised to maximum fluorescence value within each scan to obtain relative fluorescence. All the measurements were performed in triplicate. The T_m value represents the flection point of the transition curve, as described by the Boltzmann equation.[28]

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2.2 Additional information

The following experiment was also conducted as part of the published study, but was not included in the manuscript.

2.2.1 Generation of *S*-GlcNAc 1-thioacetate through the use of an α-TGL

2.2.1.1 Evaluation of potassium thioacetate as the thiol acceptor molecule

We decided to test if potassium thioacetate can act as the thiol acceptor in an α-TGL-catalyzed reaction because of its low pK_a and because hydrolysis of the resulting thioester product would give 1-mercapto-α-GlcNAc, a useful starting material in several syntheses. The α-*N*-acetyl-thioglycosaminoligases *Cp*GH89-E483Ala/Ser/Gln were chosen as the α-TGL and 2NP/2,4DNP-α-GlcNAc as donors for these tests (Figure 2.1). The thioglycoligation reactions performed at different pH values (5.8-8.0) using 100 mM potassium thioacetate were evaluated using TLC analysis. A new spot (which could correspond to the expected product) was observed only in the presence of the α-TGLs (Figure 2.2). The spot was more visible in the reactions conducted at pH values between 5.8 and 7.3. The reactions performed with α-GlcNAc-DNP as donor showed more hydrolysis of the donor, especially with *Cp*GH89-E483Gln, while those performed with α-GlcNAc-ONP still showed a lot of unreacted donor being present. This indicated that the reactions with α-GlcNAc-ONP required longer incubation than those with α-GlcNAc-DNP, especially when *Cp*GH89-E483Gln is used as α-TGL, or *Cp*GH89-E483Ala or *Cp*GH89-E483Ser at pH 5.8-6.5.

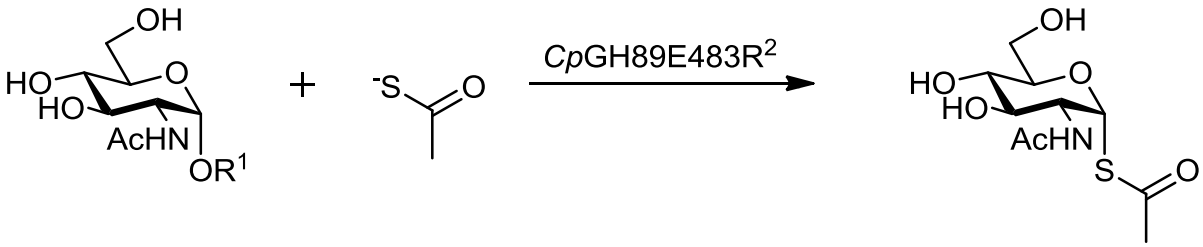


Fig. 2.1: The enzymatic reaction of α-*N*-acetyl-thioglycosaminoligases *Cp*GH89-E483Ala/Ser/Gln with 2NP/2,4DNP-α-GlcNAc as donors and potassium thioacetate as acceptor and the excepted product. R^1, 2,4DNP/2NP-α-GlcNAc and R^2, E483Ala/Ser/Gln

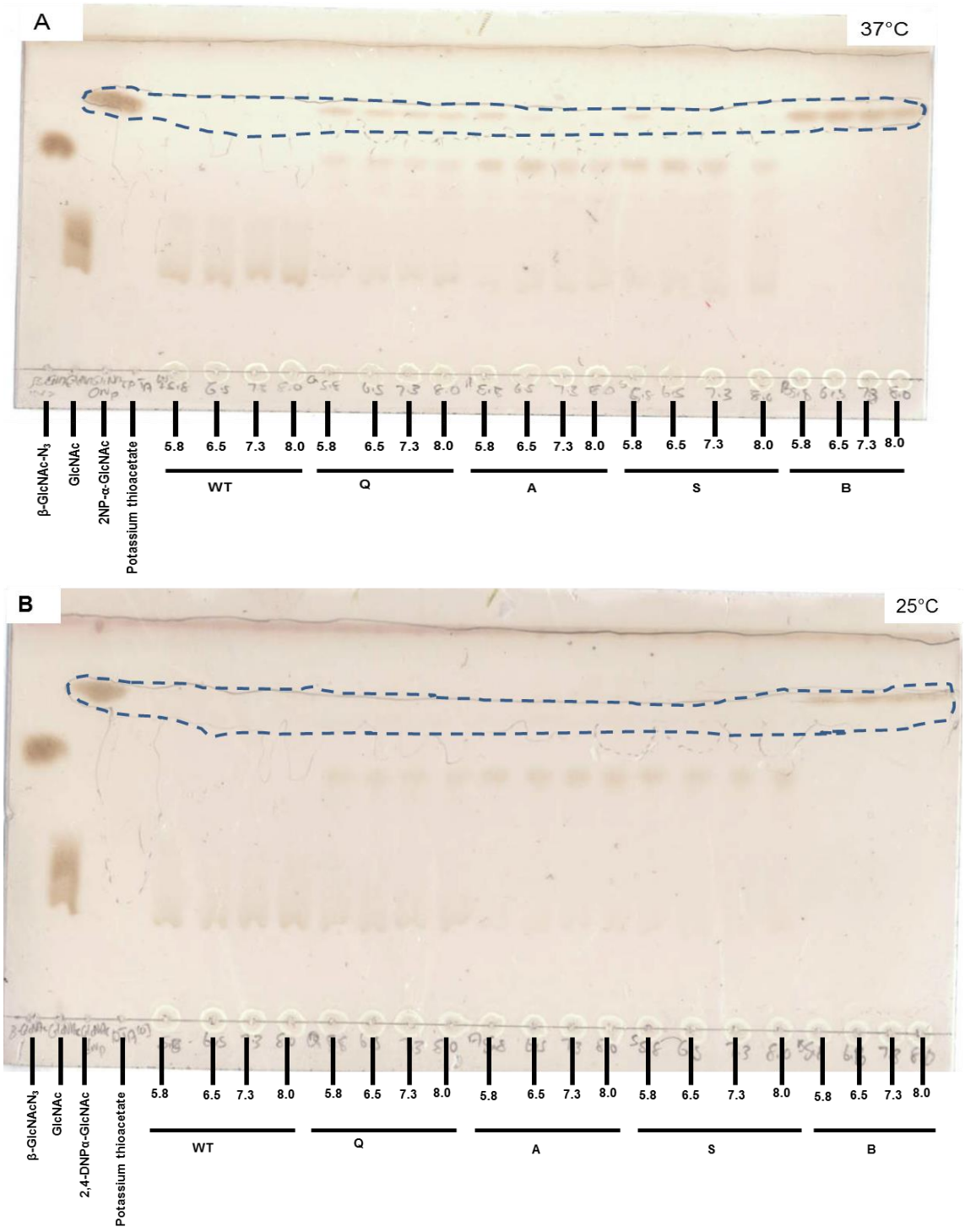


Fig. 2.2: TLC analysis of α-*N*-acetyl-thioglycosaminoligase reaction with 100 mM of potassium thioacetate as acceptor and 2,4DNP/2NP-α-GlcNAc as donors at increasing pH concentrations (pH 5.8-8.0). WT, *Cp*GH89-wild type; A, *Cp*GH89-E483A; S, *Cp*GH89-E483S; Q, *Cp*GH89-E483Q and B, no enzyme. **A**, α-GlcNAc-ONP, **B** α-GlcNAc-DNP.

2.2.1.2 Preparative scale biocatalytic preparation of S-GlcNAc 1-thioacetate using the α-TGLs CpGH89-E483Ala/Ser/Gln

To establish if the spot observed in the TLC analysis of the small scale reactions done with potassium thioacetate as acceptor indeed corresponds to the expected product, preparative scale biocatalysis was performed to allow for isolation of the product. The biocatalysis reactions were performed using *Cp*GH89–Glu483Ala and/or Ser using ~0.08 mmol 2NP-α-GlcNAc as donor, buffer with pH 7.3, and 20-fold molar excess acceptor at 37°C. The reaction progress was monitored by TLC until completion, that is, until depletion of the donor. After the completion the enzyme was removed as described for the other preparative scale reactions. However, during purification process the new spot identified during the test reactions disappeared, with the signal of the by-product (GlcNAc) spot getting stronger. This suggested that during removal of the buffer components conditions changed such that the thioester bond of the expected product was cleaved, releasing 1-mercapto-α-GlcNAc, which could have more or less running TLC condition as GlcNAc. Unfortunately, separation of the degradation product and GlcNAc the by-product proved to be very difficult, so that it was impossible to verify the structure of the degradation product. This led to investigation of reactions with potassium thioacetate as acceptor to be abandoned.

Chapter 3:
Chemo-enzymatic synthesis of GlcNAc thioglycosides and 'click' adducts as alternative substrates or inhibitors of the deacetylases MshB and BshB

3.1 Introduction

The problem of antibiotic resistance in *Staphylococcus aureus* (*S. aureus*) and *Mycobacterium tuberculosis* (*M. tuberculosis*) is increasing rapidly [1-7], with millions of people dying every year as a result of infections by these pathogens. Additionally, very few new antibacterial drugs have been developed in the past decades. Consequently, the world is heading towards a post-antibiotics era [5].

The mycothiol (MSH) and bacillithiol (BSH) biosynthetic pathway enzymes and enzymes dependent on these metabolites are currently viewed as potential targets for the development of new antibacterial drugs against *M. tuberculosis* and *S. aureus*, respectively [8-11]. MSH and BSH are low molecular weight thiols that play several essential functions within their hosts [8, 10, 12-15]. Apart from the main function of maintaining the redox balance in the microorganism, they can also provide nutrients during starvation, or act as enzyme co-factors. In addition, they are utilised by the microorganisms in defensive reactions against xenobiotic agents, including antibiotics.

The range of reactions are catalysed by different MSH-dependent enzymes which includes mycothiol *S*-transferase (MST), mycothiol disulphide reductase (Mtr), thiol peroxidase, mycothiol-*S*-nitrosoreductase/-formaldehyde dehydrogenase (MscR) and mycothiol *S*-conjugate amidase (Mca (Figure 3.1). The metabolic reactions of BSH are not yet fully characterized, but are assumed to occur in the same way as MSH [10, 12, 15]. For example, the BSH-dependent detoxification metabolism has been found to occur in the same way with MSH, including the involvement of enzymes such as bacillithiol *S*-transferase (BST), with exception of the additional acetylation by cysteinyl *S*-conjugate acetyltransferase (CAT) of the cysteinyl *S*-conjugate released by the action of bacillithiol *S*-conjugate amidase (Bca) (compare

Figure 3.2 to Figure 3.1) [12, 15, 16]. Of the different MSH and BSH biosynthetic- and -dependent enzymes, the deacetylases BshB, MshB, Mca and Bca are specifically considered good potential targets for the development of new antimicrobial drugs. This is because their functions are interlinked and because compounds developed as inhibitors of MshB/Mca have been found to inhibit both enzymes. The enzymes are also interlinked because in the absence of the *mshB* and *bshB* genes, Mca and Bca allow for the synthesis of MSH and BSH. This was demonstrated by a gene knockout study in which the *mshB* gene was removed; the resulting strain was found to still produce MSH, even in the absence of *mshB* [8, 17]. Mca normally hydrolyses the glucosaminyl-amide bond of the MS-conjugated complex formed between MSH and a xenobiotic during the elimination of such xenobiotics; the result is a mercapturic acid derivative and glucosamine-inositol (GlcN-Ins), a product of MshB (Figure 3.1). The generated GlcN-Ins can then be recycled back to the MSH biosynthetic pathway to generate MSH, while the mercapturic acid derivative is excreted by the microorganism. The same reaction sequence is thought to occur with Bca (Figure 3.2). In addition, these metalloamidases have also been attributed to the development of drug resistance within the respective bacteria [8, 15, 16].

3.1.1 Functions of BshB, MshB, Bca and Mca

BshB, MshB, Bca and Mca are enzymes that belong to the LmbE-like superfamily of metallohydrolases that catalyse the breakdown of several biological substrates in the presence of a metal ion such as zinc, cobalt, magnesium etc., with zinc usually being the preferred metal [11]. Mca and Bca are metalloamidases that hydrolyse the *N*-acyl group of compounds with an *N*-acylglucosamine core, while BshB and MshB are metallodeacetylases that hydrolyses substrates with an *N*-acetylglucosamine core [11, 15, 18]. Mca and Bca hydrolyse the glucosaminyl-amide bond of MS-S-conjugate and Bs-S-conjugate, respectively to release mecapturic acid toxin and their relative GlcN-moiety: GlcN-Mal for Bca and GlcN-Ins for Mca which are recycled back into the biosynthetic pathway. BshB catalyses the second reaction of BSH biosynthetic pathway, while MshB catalyses the third reaction of MSH biosynthetic pathway. All four metallohydrolases reactions yield an *N*-glucosamine glycosides as products (Figure 3.1 and 3.2).

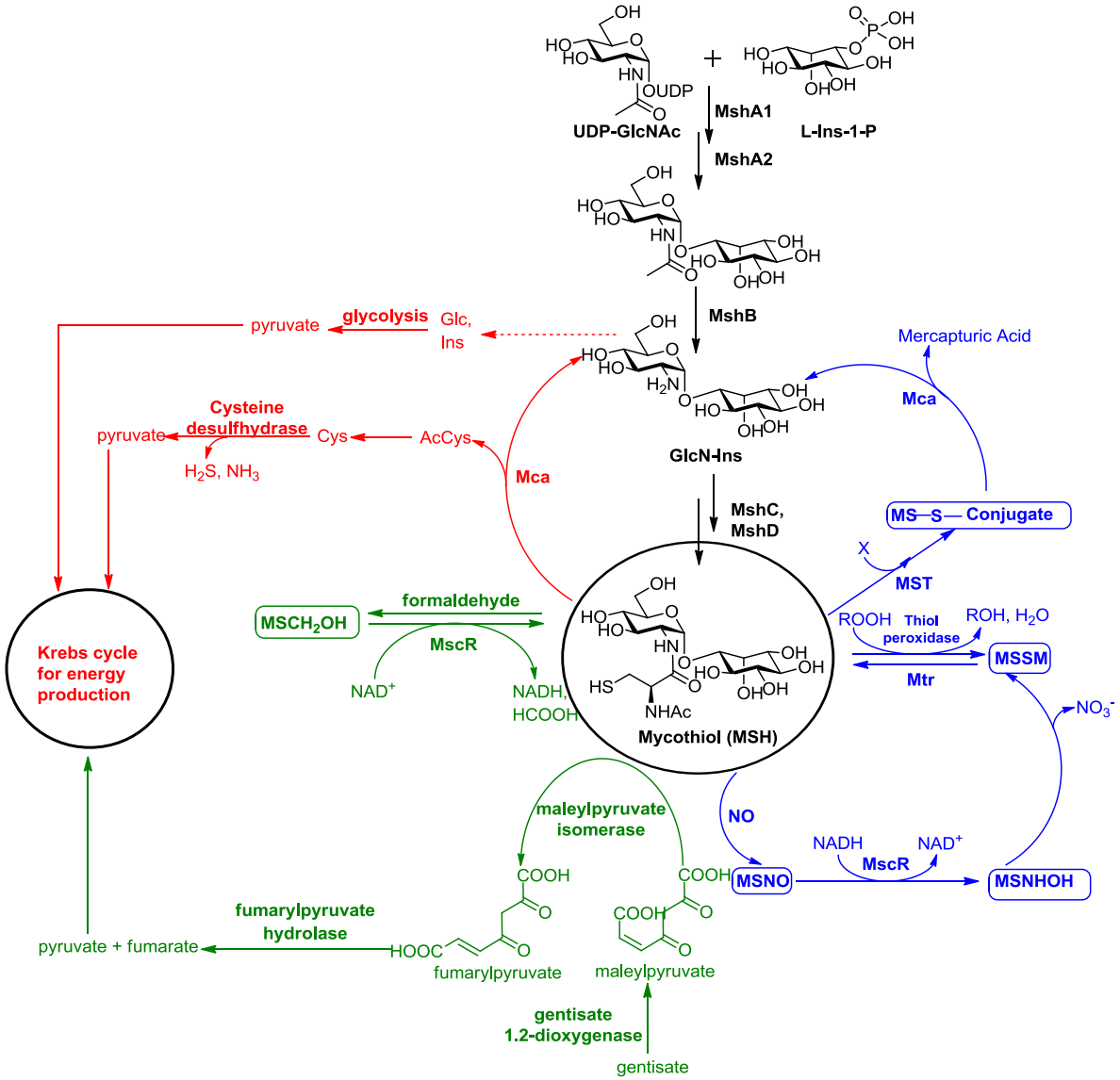


Fig. 3.1: The MSH biosynthesis pathway and the different metabolic reactions with the various MSH-dependent enzymes [8, 14, 19, 20]. The biosynthesis of MSH is represented in black, while the defensive reactions against xenobiotics are shown in blue, reaction in which MSH acts as a co-factor in green, and the degradation of MSH during nutrient starvation illustrated red. ROOH, reactive oxygen species; MSSM, mycothiol disulphide; NO, nitrogen reactive species; $MSCH_2OH$, *S*-hydroxymethyl-mycothiol; MSNO, nitrosomycothiol; MSNHOH, mycothiol-*N*-hydroxy-sulfenamide and X, xenobiotics.

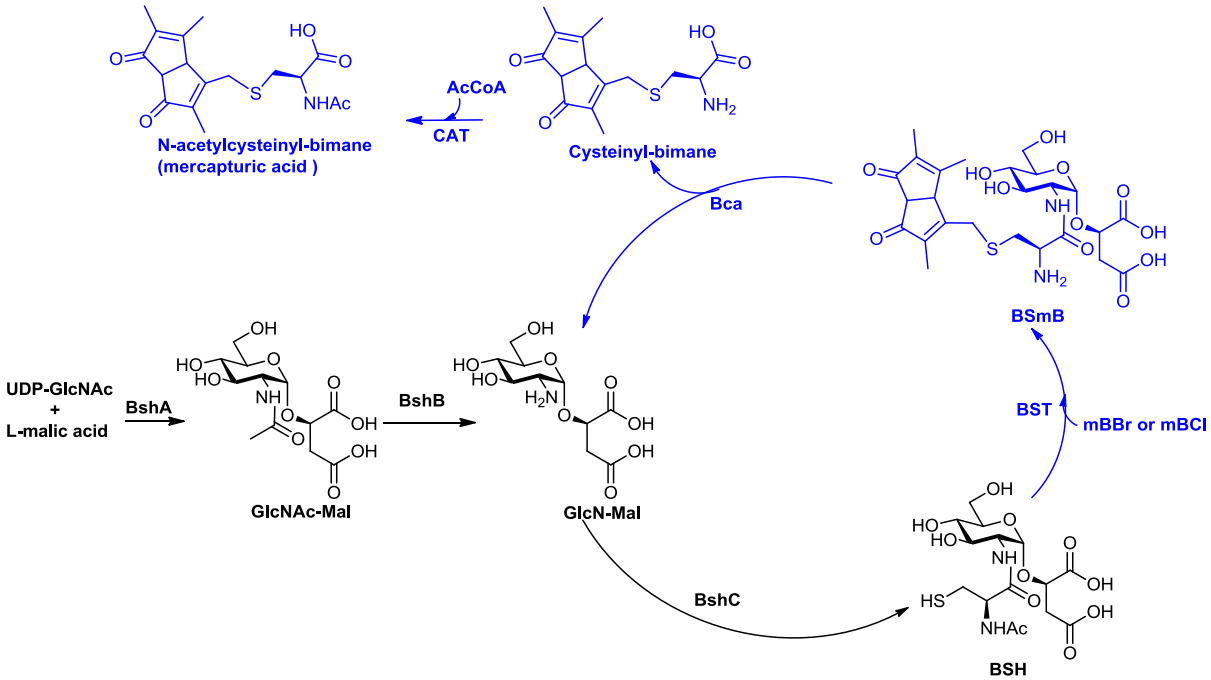


Fig. 3.2: The BSH biosynthesis pathway (black) and the detoxification reactions with the various BSH-dependent enzymes (blue) [12]. BST, bacillithiol *S*-tranferase; Bca, bacillithiol *S*-conjugate amidase; CAT, cysteinyl *S*-conjugate acetyltransferase and BSmB, bacillithiol-S-bimane.

The crystal structures of BshB [21] and MshB [22-24] have been resolved with that of Bca and Mca still pending. MshB is also the enzyme for which its catalytic mechanism has been studied the best. MshB was found to utilise an acid-base mechanism (i.e. a carboxypeptidase-like mechanism) during the deacetylation of its *N*-acetylglucosamine substrate (Figure 3.3) [11, 25]. In this mechanism the metal is attached to Asp 16, His13 and His147 of MshB, with Asp15 and His144 acting as base and acid-residues during catalysis, respectively. The metal promotes the deprotonation of a water molecule by Asp15 to generate the nucleophile that attacks the acetamido group's carbonyl oxygen. The resulting oxyanion intermediate is stabilized by interactions with the metal and with Tyr142. The His144 enables collapse of the tetrahedral intermediate, resulting in the deacetylation of the substrate. The catalytic mechanisms of Bca, Mca and BshB are not fully understood, but are assumed to be similar to MshB, since all four enzymes contain an *N*-acetyl-D-glucosaminylphosphatidylinositol deacetylase domain within their active site [11].

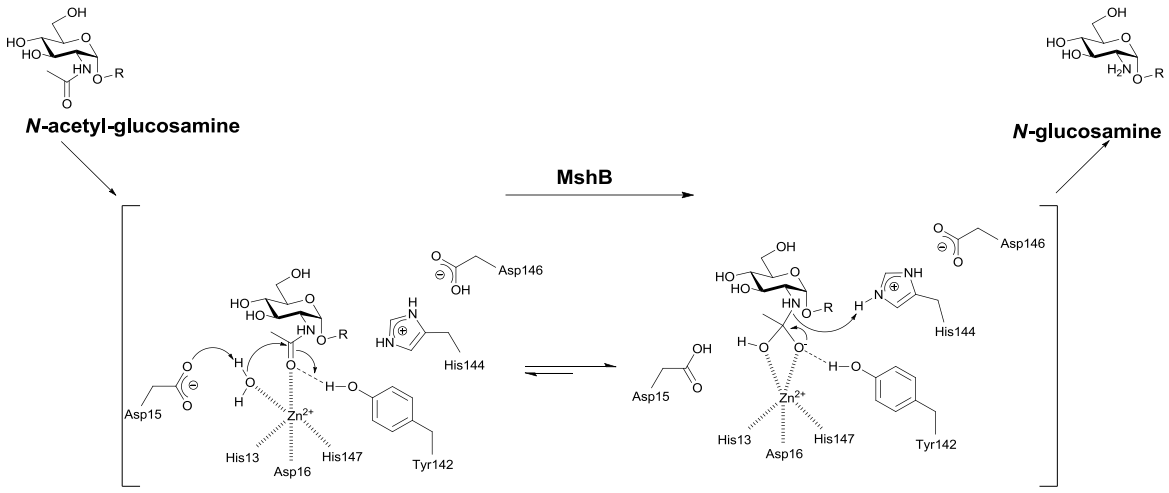


Fig. 3.3: The catalytic mechanism of MshB [25]

3.1.2 Metallohydrolases as targets for drug development

The original research on MSH biosynthetic or MSH-dependent enzymes as potential drug targets was done on Mca as this was the first of these enzymes to be discovered [26-29]. Mca was found to be inhibited by a number of bromotyrosine alkaloids from marine sources, as well as by synthetic compounds with the same scaffold as the natural products [27]. As a result of the lack of a Mca crystal structure most studies has since focussed on MshB for which crystal structures are available [22-24].

The first MshB inhibitor compounds were chemically synthesised in 2007 by Meteferia *et al.*, wherein 23 analogues of MSH were generated and divided into three groups: sulphonamides, amides and heterocycles [30]. The heterocycles showed the most inhibition, with the same compounds also being found to inhibit Mca. Another three groups of MshB inhibitors were synthesised in 2010 by Gammon *et al.,* namely thioglycosides, cyclohexyl-deoxy-2-*C*-alkyglucosides and redox cyclers consisting of plumbagin tethered with methylene carbons and an amide connected to phenyl-2-deoxy-2amino-1-thio-α-D-glucopyranoside [31]. Again, these compounds were found to inhibit both MshB and Mca, with redox cyclers being the most potent. As such one can anticipate that developing antimicrobial drugs against the one protein would also target the other one, i.e. hitting two birds with one stone. However, the problem with the inhibitor compounds that are currently available is that they inhibit the enzymes only at micromolar concentrations; this is not reasonable for the development of new anti-bacterial drugs.

The research on BSH biosynthetic- and -dependent enzymes has only been gaining more attention recently after the identification of BSH in 2009 by Newton *et al* as the other low molecular weight thiol found in *S. aureus* and Firmicutes such as *Bacillius* species and *Clostridium* amongst others [15, 32]. Gene knock-out studies of BSH biosynthetic enzymes showed that BSH is essential for the stress resistance, even though some of the bacteria have low molecular weight thiol such as cysteine and CoA, in the case of *S. aureus* [10, 15, 16]. The mutation of the *bshA* gene was found to delay the growth of the bacteria, while that of *bshB* and *bshC* genes resulted in loss of BSH production [12, 15, 33-36]. No studies on the inhibition of the BSH biosynthetic pathway enzymes or dependent enzymes have been reported to date.

3.1.3 Problem identification and rationale

The biggest challenge affecting the development of inhibitors of the MSH and BSH biosynthetic enzymes is the lack of availability of their natural substrates or alternative substrate in sufficient quantity to support inhibitor screening efforts. The natural substrate of MshB, α-*N*-acetyl-glucosamine-inositol (α-GlcNAc-Ins), is currently generated either by chemo-enzymatic manipulation or by chemical synthesis. The chemo-enzymatic process involves treatment of mycothiol-*S*-conjugates isolated from natural sources with Mca to release GlcN-Ins [37], while the chemical synthetic process requires multiple steps [28, 31, 38-40]. The problem with both these methods is the low final yield of the product obtained after the lengthy preparation process. Consequently, several GlcNAc-based glycosides, GlcNAc and thioglycosides have been chemically synthesised and tested as alternative substrates of MshB [18, 31, 41-44]. However, these compounds often also require a lengthy process to be generated, give low yields for the final product and also have shown solubility problems in aqueous environments; examples include 2,4DNP-α-*S*-GlcNAc and α-GlcNAc-SPh [31, 41]. Furthermore, the alternative substrates with high specific activity in relation to the natural substrate are the ones with amidase activity [18]. The problem with amidase activity is that it does not really mimic what happens with the natural substrate at the active site of MshB, since MshB natural activity is the deacetylase activity, as such one cannot really use them during inhibitor studies. Therefore GlcNAc itself is still the most used alternative MshB substrate, also because it is commercially available. However, is shows very low deacetylation activity [18, 37, 41].

On the other hand α-*N*-acetyl-glucosamine-malic acid (α-GlcNAc-Mal), natural substrate of BshB, is generated by chemical synthesis over several steps that involve the protection and deprotection of the intermediate compounds [13, 45]. Several compounds with the GlcNAc moiety have also been chemically synthesised and tested as alternative substrate of BshB [46]. Unfortunately these alternative substrates displayed low activity in relation to α-GlcNAc-Mal. The relatively recent discovery of BSH means that all its other biosynthetic enzymes still require more characterisation to best understand their respective functions. Also in their case the absence of substrates or suitable substrate analogues hinders such full characterisation, which also limits full inhibition studies.

In a recent study by *Tshililo et al.* an α-*N*-acetylglucosaminidase (*Cp*GH89) from *Clostridium perfringens* was converted into α-*N*-acetyl-thioglycosaminoligase which allowed the biocatalytic production of α-GlcNAc thioglycosides, as well as α-GlcNAc-N_3 on multimilligram scale [see publication reproduced as Chapter 2 of this dissertation]. This was achieved through the use of glycosyl donors such 2,4DNP-α-GlcNAc and 2NP-α-GlcNAc and various thiol molecules or sodium azide as acceptors. In the current study, we set out to apply this biocatalyst in the chemo-enzymatic synthesis of α-GlcNAc thioglycosides as alternative substrates of MshB and/or BshB. In addition, the α-GlcNAc-N_3 prepared in the previous study was used as substrate in 'Click'-chemistry reactions to generate several GlcNAc-linked triazoles. 'Click'-chemistry describes a group of high yielding chemical reactions that are easily performed and that require no or minimal purification of the final product, among other factors [47]. Included among such reactions are alkyne/azide cycloaddition and nucleophilic ring-opening reactions, carbonyl chemistry of the non-aldol type and additions of carbon multiple bonds. Of these four reactions alkyne/azide cycloadditions that leads to the formation 1,4-substituted 1,2,3-triazoles are the most widely used. Its mechanism has been described as an annealing step-wise reaction which occurs through an intriguing six membered copper-containing intermediate III [47-49]. 'Click'-chemistry has been mostly applied in the drug discovery, in the pharmaceutical industries, and in polymer science among other fields. In this study the alkyne/azide cycloaddition reaction was applied to chemically synthesise GlcNAc-linked triazoles as potential alternative substrates of MshB and BshB.

The α-GlcNAc-based glycosides together with the GlcNAc-linked triazole compounds and various inhibitor compounds generated by the Gammon group from the University of Cape Town, were subsequently characterised as either substrates or inhibitors of MshB from *M. tuberculosis*.

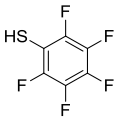
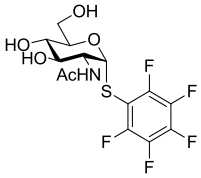
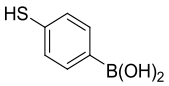
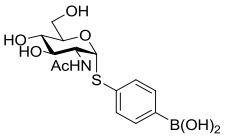
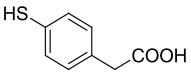
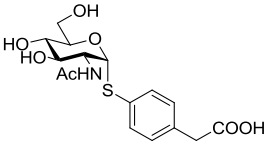
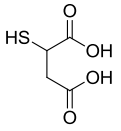
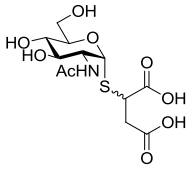
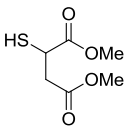
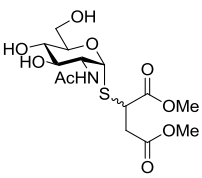
3.2 Results and Discussion

3.2.1 The assessment of α-*N*-acetyl-thioglycosaminoligases with several thiol-compounds

In 2011, Almendros *et al.* found that the thioglycoligation reaction of furanothioglycoligase only took place with aryl thiols which had a pK_a value between 5.83 and 8.75, indicating that the pK_a value of a thiol group influenced its ability to act as an acceptor during the reaction [50]. This was also observed by Tshililo *et al.*, that is, only thiol-containing molecules with pK_a values between 6.6 and 8.1 were able to act as thiol-acceptors during the thioglycoligation reaction catalysed by α-*N*-acetyl-thioglycosaminoligases [Chapter 2]. Consequently, based on the precedent of these two studies, several thiolated compounds with pK_a values between 3.49 and 6.0 (Table 3.1) were chosen for this study for the evaluation as thiol-acceptors for the generation of α-GlcNAc thioglycosides that could potentially act as alternative substrates for MshB and/or BshB. Apart from being commercially available the aryl thiol compounds (entry 1-6) were chosen as acceptor owing to their low *pKa* values of the thiol group and because α-GlcNAc-SPh derivates have been found to act as alternative substrate for MshB [31, 41]. Mercaptosuccinic acid, entry 7-8, was chosen as an acceptor for the generation of α-*N*-acetyl-glucosamine-thio-malic acid (α-GlcNAc-*S*Mal), an analog of BshB natural substrate α-GlcNAc-Mal. Meanwhile dimethyl mercaptosuccinate (entry 9-10) was chosen for the generation of *S*-(2-acetamido-2-deoxy-α-D-glucopyranosyl) 2-thiosuccinate (α-GlcNAc-SMal $(OMe)_2$) with the idea of chemically converting it into α-GlcNAc-*S*Mal. No thiol sugar compounds were utilised due to the fact that they are not commercially available and it is challenging to chemically synthesis them. In addition, their thiol *pKa* values limits them to act as acceptor, that is, the *pKa* values of some thiol group of thio-sugar is greater than 8, which restricts the generation of thiolate anion. An example is that of thio-sugar methyl 4-deoxy-4-thioglucoside in Chapter 2. Thio-sugar methyl 4-deoxy-4-thioglucoside was chemically synthesise through several chemical steps and in the

end it was unable to act as an acceptor due to its *pKa* value of greater than 8 [Chapter 2]. The two α-*N*-acetyl-thioglycosaminoligases (*Cp*GH89–E483A and *Cp*GH89–E483A) with the best activity in large scale reactions as described in Chapter 2 were utilised for the assessment using the same reaction conditions used in the previous study.

Table 3.1: Activity of *Cp*GH89–E483A and *Cp*GH89–E483S with various thiol-containing molecules as acceptors and 2,4DNP/2NP-α-GlcNAc as donor in all entries

Entry	Acceptor	pK_a	Expected product	*Cp*GH89 mutant	Product formation
1	 Pentafluorothiophenol	3.42		E483A	No
2				E483S	No
3	 4-Mercaptophenyl-boronic acid	5.67		E483A	No
4				E483S	No
5	 4-Mercaptophenyl-acetic acid	6.0		E483A	No
6				E483S	No
7	 Mercaptosuccinic acid	3.49	 α-GlcNAc-SMal	E483A	No
8				E483S	No
9	 Dimethyl mercaptosuccinate*	3.49	 α-GlcNAc-SMal(OMe)$_2$	E483A	Yes
10				E483S	Yes

*Chemically synthesised, see materials and methods section.

None of the three aryl thiol compounds gave products with either α-*N*-acetyl-thioglycosaminoligases, most likely due to their poor solubility in the aqueous buffer. The lack of reaction was not due to inhibition of the enzyme, since the hydrolysis of the 2,4DNP-α-GlcNAc donor was observed only in the presence of the enzymes, both wild type and mutants (see Figure 3.4). However, for the reactions with the less reactive 2NP-α-GlcNAc donor, hydrolysis activity was only observed in the presence of the wild type enzyme.

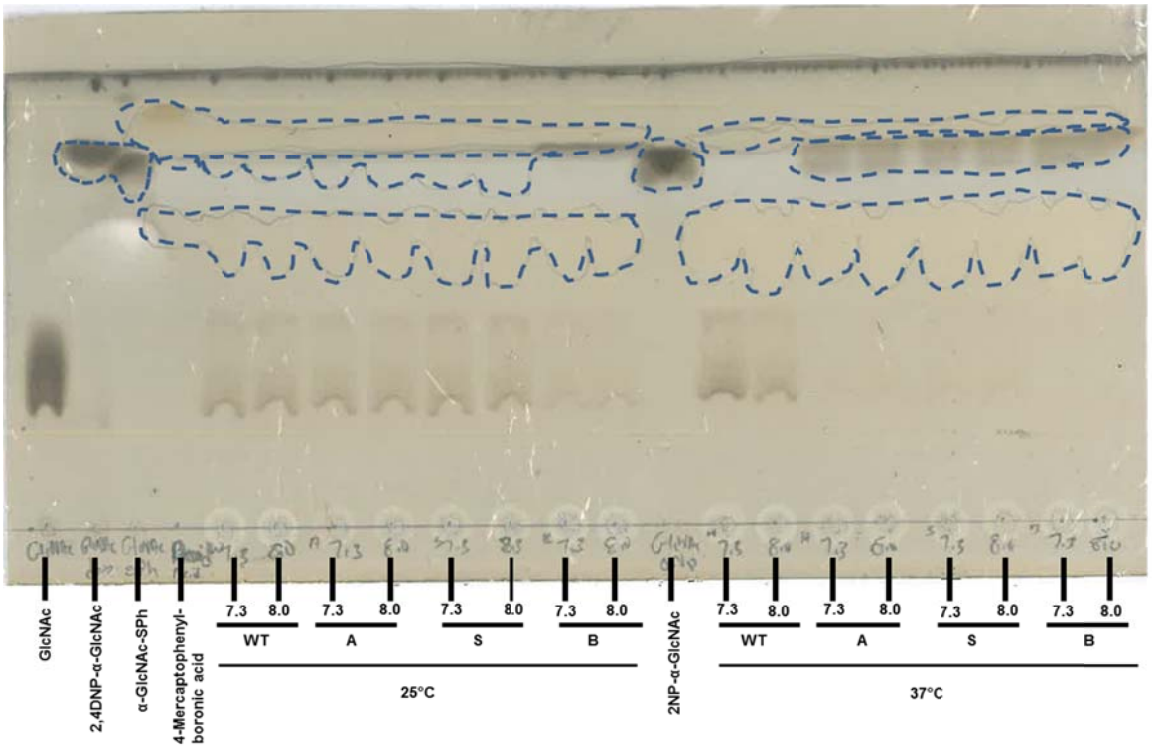


Fig. 3.4: TLC analysis of the reaction of the α-*N*-acetyl-thioglycosaminoligases with 4-mercaptophenyl-boronic acid as acceptor and 2,4DNP-α-GlcNAc or 2NP-α-GlcNAc as donors. α-GlcNAc-SPh, GlcNAc, 4-mercaptophenyl-boronic acid, 2,4DNP-α-GlcNAc and 2NP-α-GlcNAc are loaded on TLC plate as references. Reaction mixtures were incubated at either 25°C or 37°C, as indicated, with 5mM of donors as final concentration and 100 mM of 4-mercaptophenyl-boronic acid. Spots that were UV-visible are indicated by dotted circles; other spots where visualized by staining with 10% sulphuric acid in methanol, followed by incubation at 150°C for 15 minutes. WT, *Cp*GH89 wild type; A, *Cp*GH89–E483A; S, *Cp*GH89–E483S, B, blank (no enzyme), 7.3 and 8.0 is the pH value.

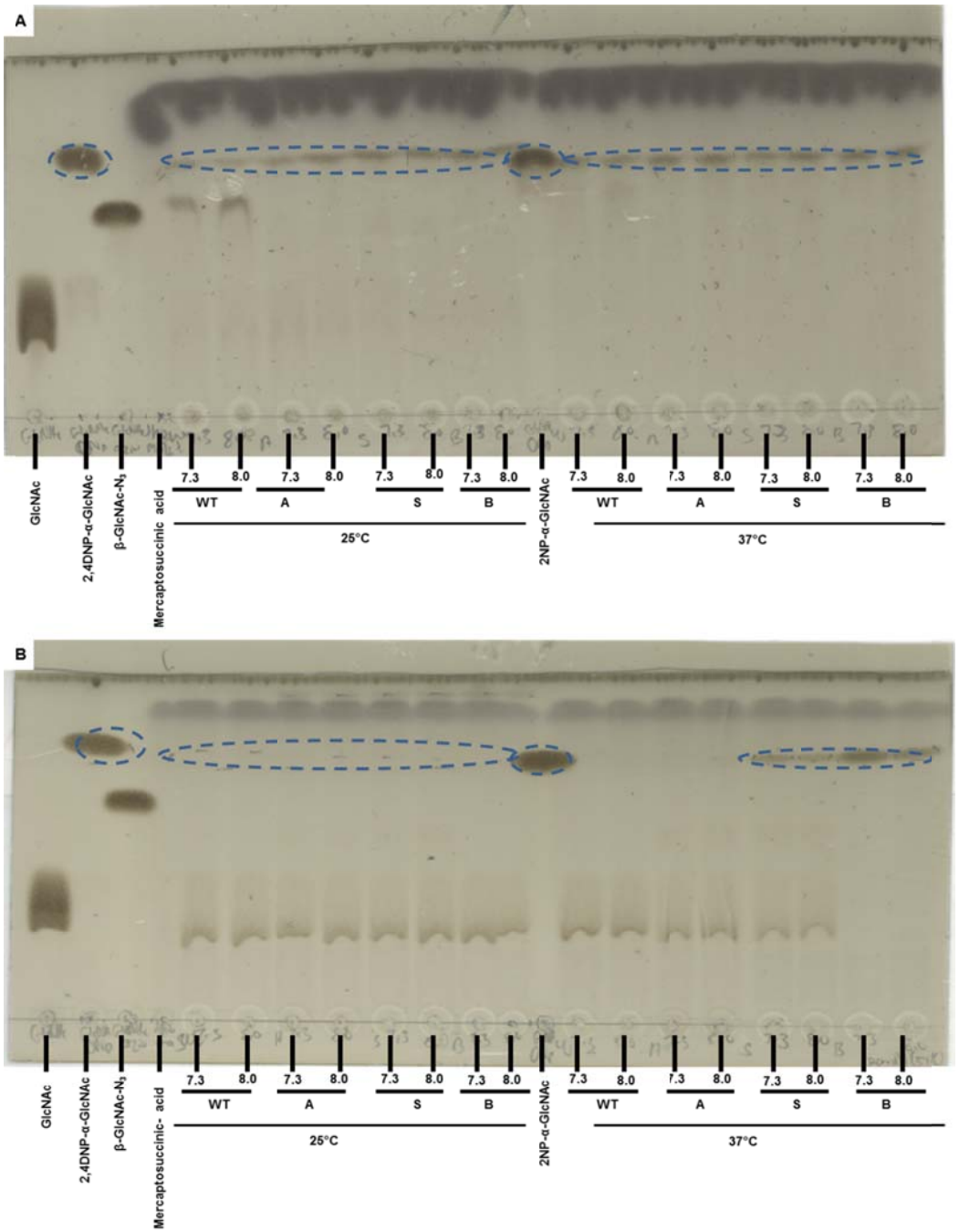


Fig. 3.5: TLC analysis of α-*N*-acetyl-thioglycosaminoligases reaction with mercaptosuccinic acid as acceptor and 2,4DNP-α-GlcNAc and 2NP-α-GlcNAc as donors. β-GlcNAc-N_3, GlcNAc, mercaptosuccinic acid, 2,4DNP-α-GlcNAc and 2NP-α-GlcNAc are loaded on TLC plate as references. Reaction mixtures were incubated at either 25°C or 37°C, as indicated, with 5mM of donors as final concentration and various concentration of mercaptosuccinic acid. **Panel A** is 100 mM of mercaptosuccinic acid as final concentration and **panel B**, 20 mM of mercaptosuccinic acid as final concentration. Spots that were UV-visible are

indicated by dotted circles; other spots where visualized by staining with 10% sulphuric acid in methanol, followed by incubation at 150°C for 15 minutes. WT, *Cp*GH89 wild type; A, *Cp*GH89–E483A; S, *Cp*GH89–E483S, B, blank (no enzyme), 7.3 and 8.0 is the pH value.

Mercaptosuccinic acid was also unable to promote thioglycoligation reaction nor hydrolysis at high concentration (100 mM), indicating inhibition of the enzymes (Figure 3.5A). However, when the concentration of mercaptosuccinic acid was reduced (i.e. from 100 mM to 5–30 mM) only hydrolysis of the donors was observed in the present of the enzymes (Figure 3.5B).

In addition, altering the final concentration of the buffer (50-500mM) with the different low concentrations of mercaptosuccinic acid also resulted only in the hydrolysis of the donors. Consequently, the expected product (α-GlcNAc-SMal, see Table 3.1) was not formed under any of the tested conditions. The incapacity of mercaptosuccinic acid to act as an acceptor at low concentration is assumed to because that maybe the α-*N*-acetyl-thioglycosaminoligases do not prefer negative charged molecules in their active site. To test such theory the carboxylic acid groups of mercaptosuccinic acid were converted into an ester producing dimethyl mercaptosuccinate thiol compound (entry 9 and 10).

The thioglycoligation reaction with 100 mM of dimethyl mercaptosuccinate thiol compound as an acceptor formed a new spot only in the presence of the two α-*N*-acetyl-thioglycosaminoligases as observed through TLC analysis (Figure 3.6). The strong signal of a new spot as observed through human eyes was formed in the reactions in which 2NP-α-GlcNAc was used as donor. The signal of new spot of reactions with 2,4DNP-α-GlcNAc was weak in relation to 2NP-α-GlcNAc. In addition, the reaction with 2,4DNP-α-GlcNAc also resulted in high hydrolyse activity of the donor. The outstanding synthetic effectiveness of this reaction encouraged us to determine if the α-*N*-acetyl-thioglycosaminoligases activity would also translate to preparative scale, and allow the biosynthesis and purification of dimethyl *S*-(2-acetamido-2-deoxy-α-D-glucopyranosyl) 2-thiosuccinate (α-GlcNAc-SMal$(OMe)_2$).

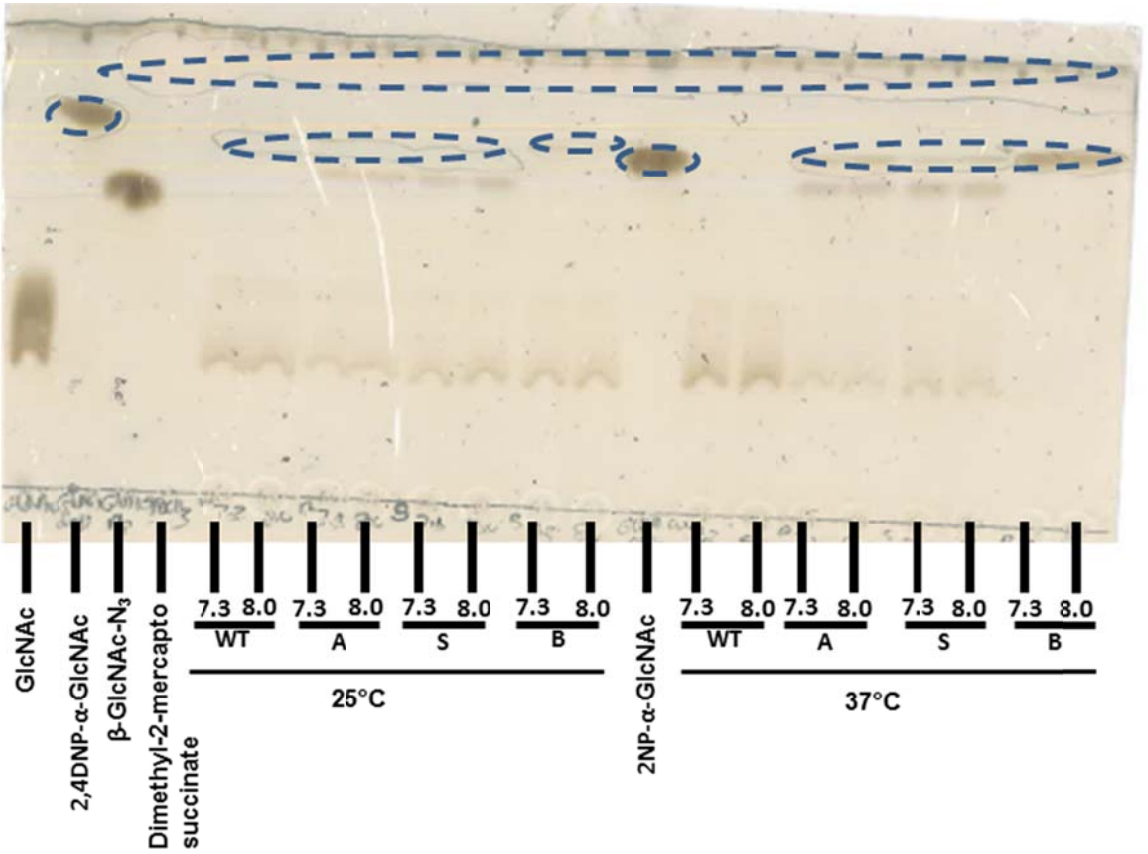


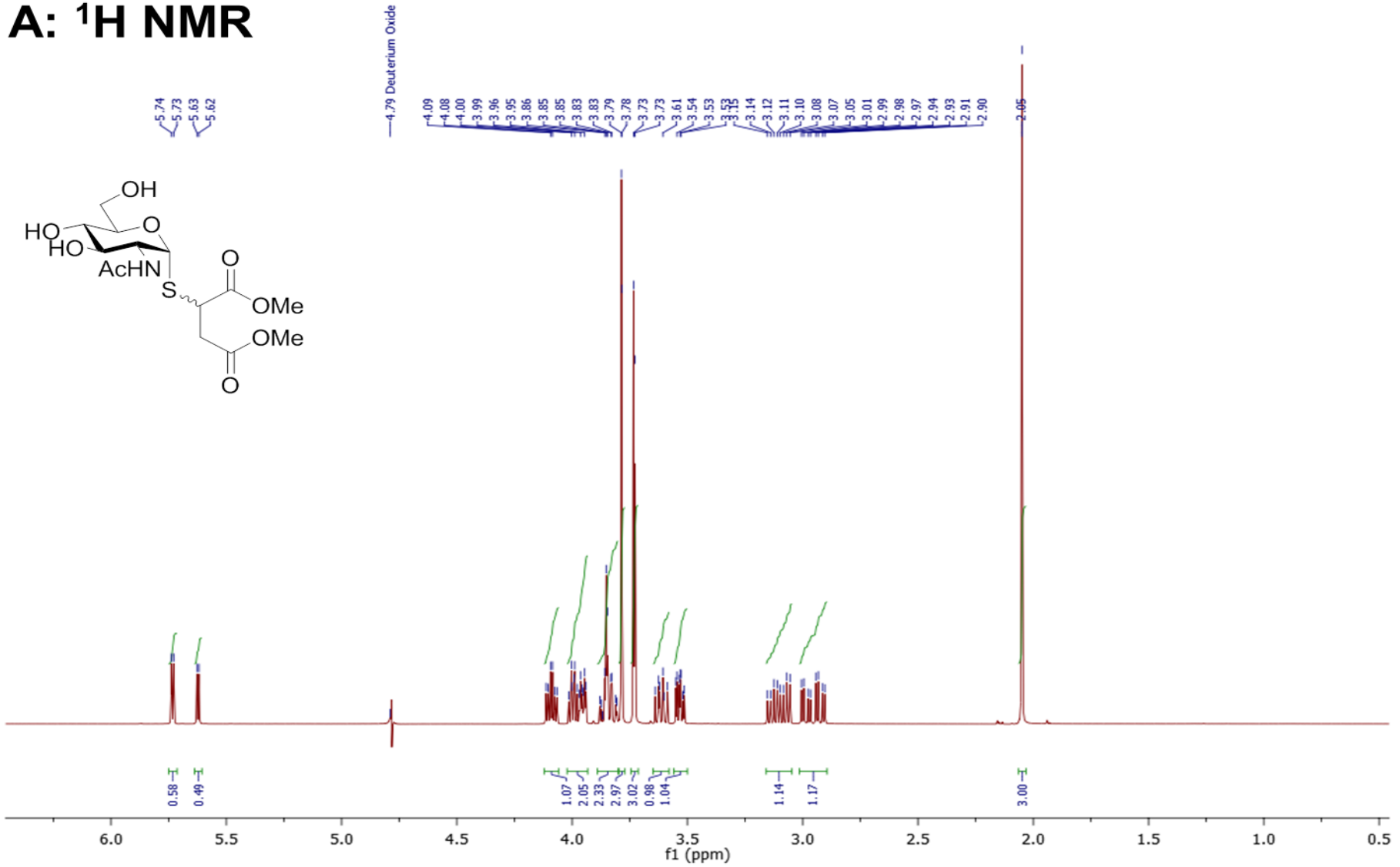
Fig. 3.6: TLC analysis of α-*N*-acetyl-thioglycosaminoligases reaction with dimethyl 2-mercaptosuccinate as acceptor and 2,4DNP-α-GlcNAc and 2NP-α-GlcNAc as donors. β-GlcNAcN_3, GlcNAc, dimethyl 2-mercaptosuccinate, 2,4DNP-α-GlcNAc and 2NP-α-GlcNAc are loaded on TLC plate as references. Reaction mixtures were incubated at either 25°C or 37°C, as indicated, with 5 mM of donors as final concentration and 100 mM of dimethyl 2-mercaptosuccinate. Spots that were UV-visible are indicated by dotted circles; other spots where visualized by staining with 10% sulphuric acid in methanol, followed by incubation at 150°C for 15 minutes. WT, *Cp*GH89 wild type; A, *Cp*GH89–E483A; S, *Cp*GH89–E483S and B, blank (no enzyme), 7.3 and 8.0 is the pH value.

3.2.2 Enzymatic synthesis of α-GlcNAc-S-Mal $(OMe)_2$ thioglycosides using the α-*N*-acetyl-thioglycosaminoligase-E483A derived from *Cp*GH89

The preparative scale reaction of α-GlcNAc-SMal $(OMe)_2$ from dimethyl mercaptosuccinate and 2NP-α-GlcNAc was carried using the above identified reaction conditions. After completion of the reaction, which is the complete consumption of the donor, the spot corresponding to the one observed during the evaluation of dimethyl mercaptosuccinate as acceptor (Figure 3.6) was purified and

analysed in the same manner as conducted by *Tshililo et al.* [see Chapter 2]. The NMR and MS spectra confirmed that the new observed spot is indeed α-GlcNAc-SMal(OMe)$_2$ (Figure 3.7). The generation of α-GlcNAc-SMal(OMe)$_2$ is very beneficial since it can be demethylated for the generation of α-GlcNAc-SMal, an analogue of BshB natural substrate, α-GlcNAc-Mal (see section 3.2.3).

A: ^{1}H NMR



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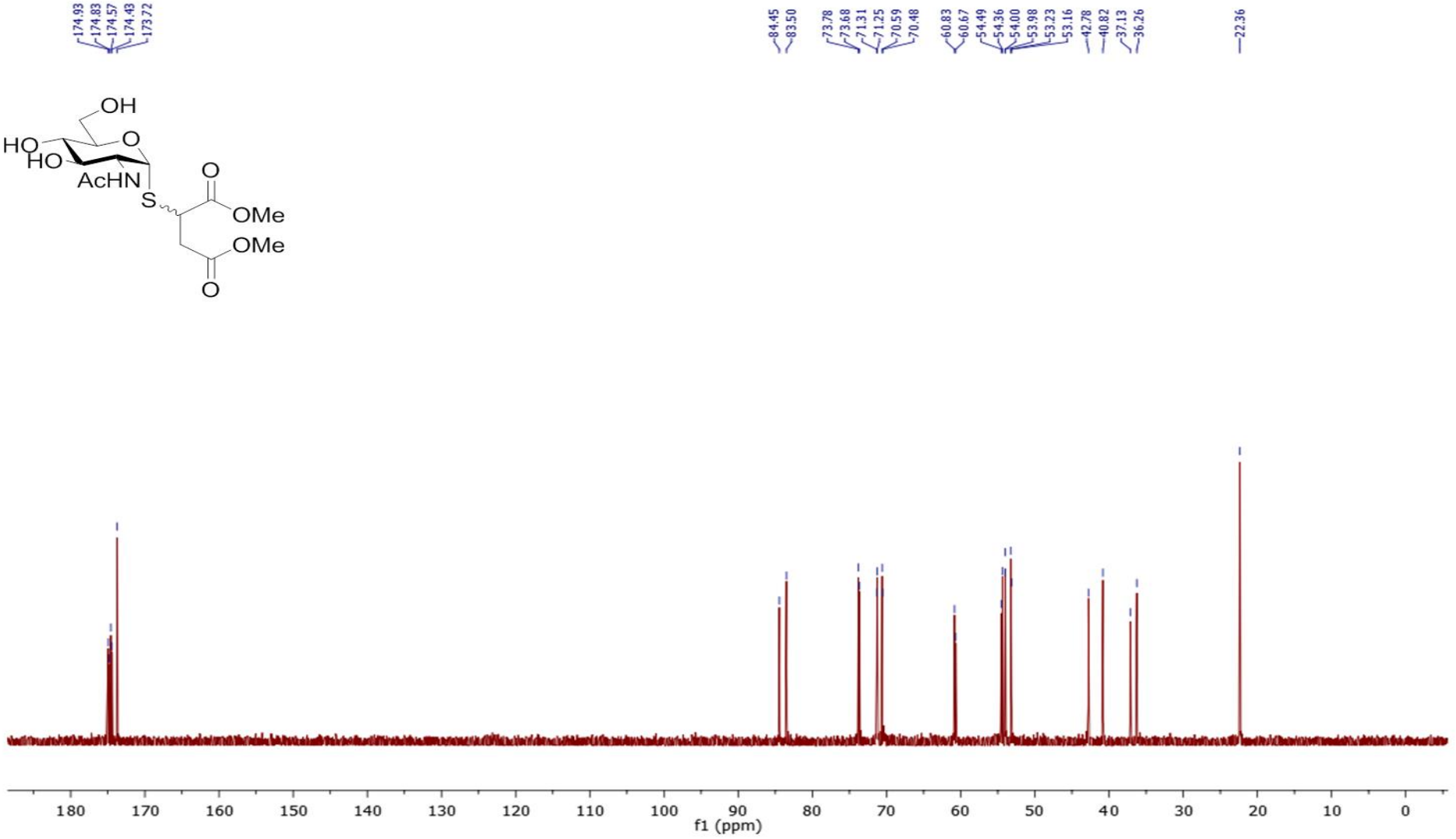
B: ^{13}C NMR



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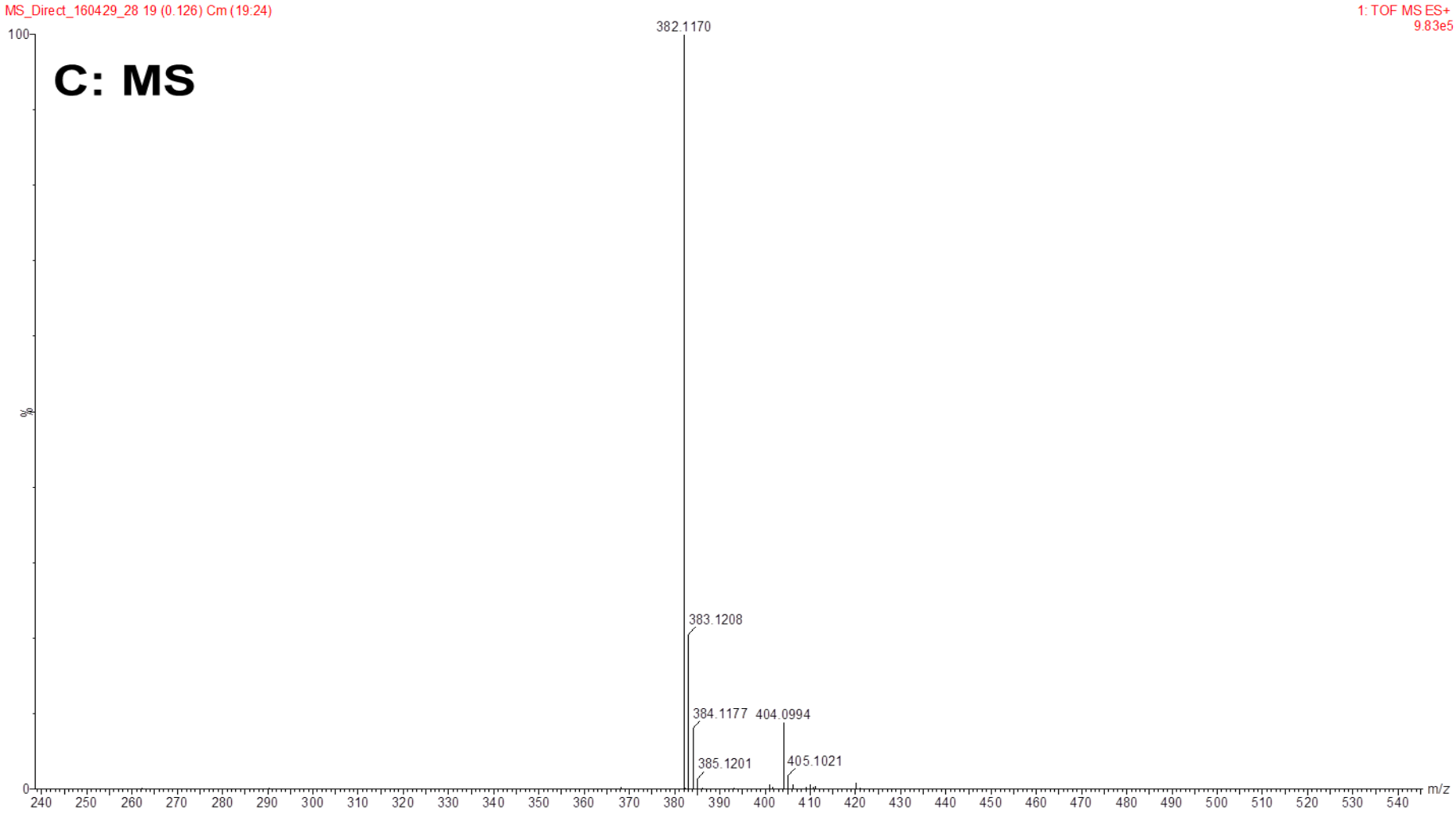


Fig. 3.7: The analysis of α-GlcNAc-SMal(OMe)$_2$ through NMR and MS. **NMR analyses:** All ^{1}H (**A**) and ^{13}C (**B**) NMR spectra were obtained using a 600 MHz Varian Unity Inova (150 MHz for ^{13}C) instruments (Varian Inc., Palo Alto, CA, USA. All chemical shifts (δ)

were referenced to the reported values of Gottlieb [51] using the signal from the residual protonated solvent for ^{1}H spectra, or to the ^{13}C signal from the deuterated solvent. Chemical shift δ values for ^{1}H and ^{13}C spectra are reported in parts per million (ppm) relative to these referenced values. All ^{13}C NMR spectra were recorded with complete proton decoupling. FID files were processed using MestraNova 6.0.2 (MestreLab Research). **MS analysis (C):** All high resolution mass spectrometry (HRMS) was performed on a Waters API Q-TOF Ultima spectrometer.

3.2.3 Chemical modification of α-GlcNAc thioglycosides obtained by biocatalysis

Since attempts at preparing α-GlcNAc-SMal by enzymatic synthesis failed, we decided to prepare this potential alternative BshB substrate by chemical synthesis using the methyl ester (α-GlcNAc-SMal$(OMe)_2$) that was successfully obtained as starting material. The same strategy was also applied to α-GlcNAc-SCH_2CO_2Me obtained by biocatalysis in a previous study (see chapter 2) to give a truncated BshB substrate analogue, α-GlcNAc-SCH_2CO_2H.

Both esters were demethylated using methods previously published by *White et al.* and *Corey et al*. in yields of between 30 and 60% (Figure 3.8) [52, 53]. In both cases the polarity of the carboxylic acid groups made purification of the compounds by flash column chromatography challenging, and was the main cause of the low yields.

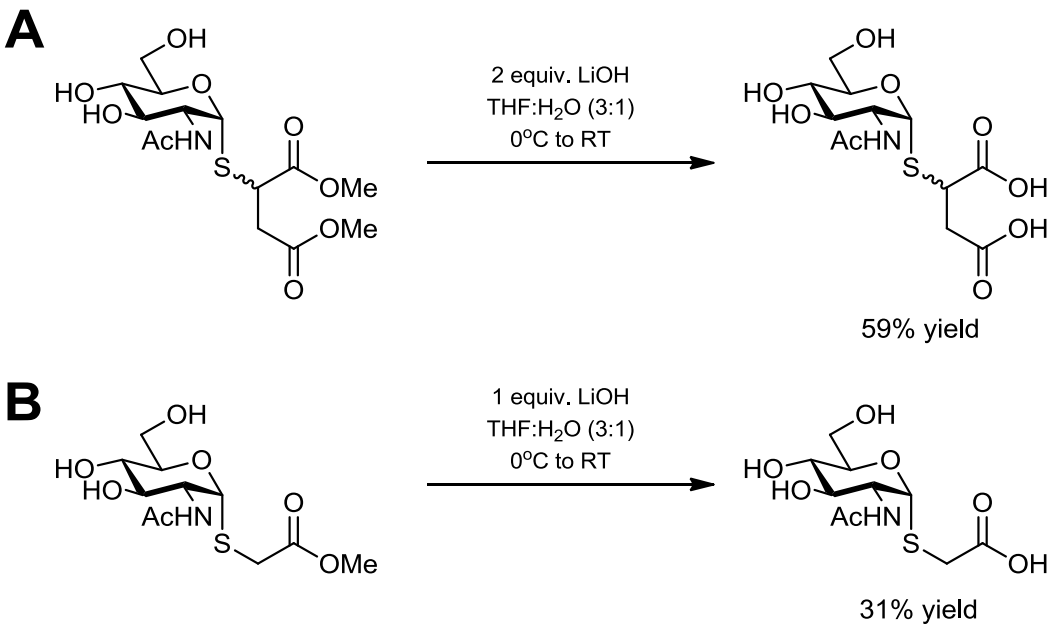


Fig. 3.8: The chemical synthesis of α-GlcNAc based thioglycosides through demethylation of enzymatically-synthesized starting materials. **Panel A**, chemical synthesis of α-GlcNAc-SMal from α-GlcNAc-SMal$(OMe)_2$ and **panel B**, chemical synthesis α-GlcNAc-SCH_2CO_2H from α-GlcNAc-SCH_2CO_2Me. **THF**, Tetrahydrofuran; **RT**, room temperature and **LiOH**, lithium hydroxide

3.2.4 Preparing GlcNAc-based "Click" adducts using α- and β-GlcNAc-N_3 as starting material

An alternative strategy to obtain MshB and BshB substrate analogues or substrate mimicking inhibitors is to use the GlcNAc core structure as scaffold and to introduce a variety of substructures in the position normally occupied by inositol or malate in these enzyme's native substrates. This can be done by using GlcNAc-N_3 to generate *N*-GlcNAc-based glycosides through their reaction with alkyne compounds by means of "Click Chemistry". Currently β-GlcNAc-N_3 is commercially available; however, to maintain the correct anomeric configuration for recognition by MshB and BshB would require the use of α-GlcNAc-N_3 instead. Fortunately, we have demonstrated that this compound can be generated by preparative scale biocatalysis from 2NP-α-GlcNAc- and NaN_3 using the α-*N*-acetyl-thioglycosaminoligase *Cp*GH89-E483A [Chapter 2], and it was therefore available for use in this study. For the Click reactions, two alkynes were chosen: propargyl alcohol and 1-ethynyl-1-cyclohexanol. The aryl alkynes were chosen base to resemble the inositol moiety of MshB and because α-GlcNAc-aryl based glycosides have found to be better alternative for MshB [31, 41]. The alcohol alkynes were selected to generate GlcNAc-triazoles compounds with alcohol group to resemble those of the inositol and malic acid moiety. Furthermore, both alkynes were commercially available, meaning no chemical synthesis required. The reactions were performed with both α- and β-GlcNAc-N_3 to investigate the importance of the anomeric configuration in the cycloaddition reaction, and subsequently in interaction with the enzymes.

Two β-GlcNAc-triazoles were generated in the presence of copper sulphate with a yield of more than 50% using previously published chemical methods by *Rostovtsev et al.* (Figure 3.9A) [48]. On the other hand, the synthesis of the α-GlcNAc-triazoles were found to be much more challenging as demonstrated by the lower yields (between 38 and 43%) that were obtained (Figure 3.9B). Additionally, Click reactions with the α-anomer required long running times to reach completion and the addition of Tris [(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA), a tertiary amine that stabilizes the copper catalyst [54]. The long reaction time and use of TBTA is assumed to be due to the steric hindrance that occurs as results of the position of the azide moiety in relation to the acetyl group at the second carbon of the GlcNAc moiety.

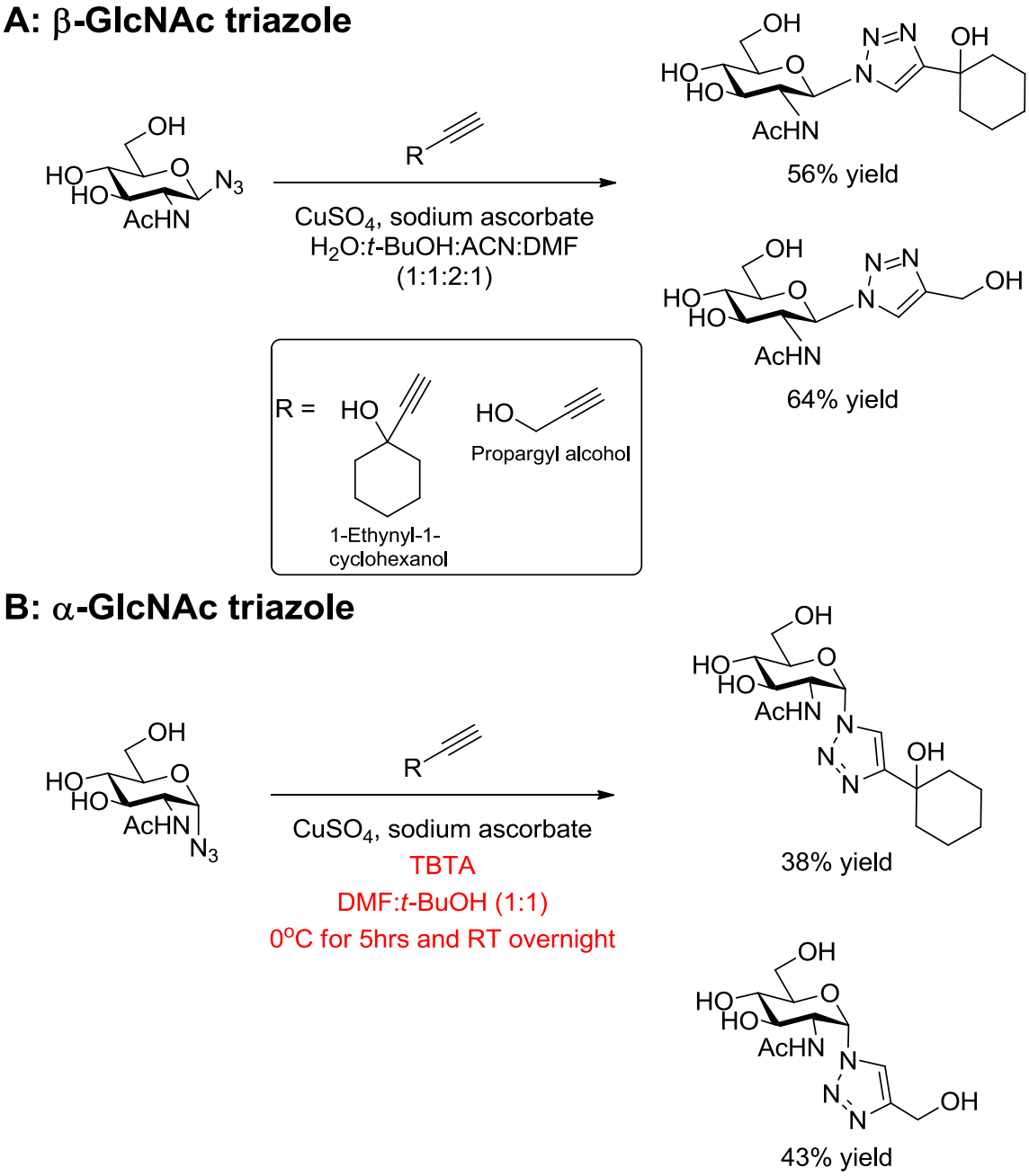


Fig. 3.9: The chemically synthesis of GlcNAc-triazoles through "Click chemistry". (**A**) Generation of β-GlcNAc-triazole from β-GlcNAc-N_3 by the method of *Rostovtsev et al.* [48](**B**) α-GlcNAc-triazoles obtained from α-GlcNAc-N_3 following the method of *Machida et al.* [54]**TBTA**, Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine; **$CuSO_4$**, copper sulphate; **ACN**, acetonitrile; **DMF**, dimethylformamide and *t*-BuOH, *tert*-Butyl alcohol.

3.2.5 Evaluation of the GlcNAc-based glycosides as potential alternative substrates of MshB

Several compounds have been chemically synthesised as alternative substrates for MshB [28, 31, 38-41]. However, the majority of them have low active in relation to the natural substrate. The chemically and enzymatically synthesised GlcNAc-based glycosides prepared in this study and previous study [Chapter 2], together with the commercial available compounds GlcNAc and β-GlcNAc-N_3 were assessed for their ability to act as substrates for MshB. The compounds were evaluated for their deacetylation-specific activity, the effect of the type of glycosidic linkage and the anomeric configuration.

A number of discontinuous assays are used to characterise MshB and BshB activity, only one continuous assay is used for characterisation of MshB activity. The discontinuous assays include 6-aminoquinolyl-*N*-hydroxysuccinimidly carbamate assay (AccQ-Fluor™) [18, 37], fluorescamine (FSA) [43] and naphthalene-2,3-dicarboxaldehyde (NDA) [46] of which all utilise derivatization agents, while the continuous assay is called a 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) base assay [41]. Due to several shortcoming of the others assays (more details in Chapter 4) the fluorescamine (FSA) derivatization assay which modifies the deacetylated amine product to give a fluorescent product was selected for these evaluations. The assay was conducted in the same way as *Huang et al*. with some modifications [43]. Since standards could not be generated for all the anticipated reaction products, glucosamine was chosen as reference to generate a standard curve to determine the amount of free amino sugar produced during each reaction as described below.

3.2.5.1 Standard curve

The standard curve was generated from the fluorescence units detected from the increasing concentration of reaction mixtures of glucosamine. The increasing concentrations reaction mixtures of glucosamine with FSA were subsequently determined by fluorimetry (FSA excitation at 395 nm and emission at 485 nm). The resulting increasing fluorescence units from the different concentration of glucosamine together with the various concentrations of glucosamine were used to plot a standard curve through which a linear equation (**y = mx + c**) was fitted (Figure 3.10). The linear equation was determined to be **y = 0.9379x + 0.8821 (R^2 = 0.9882)**,

and was used in all calculations to convert the fluorescence units detected into free amino groups produced during the reaction.

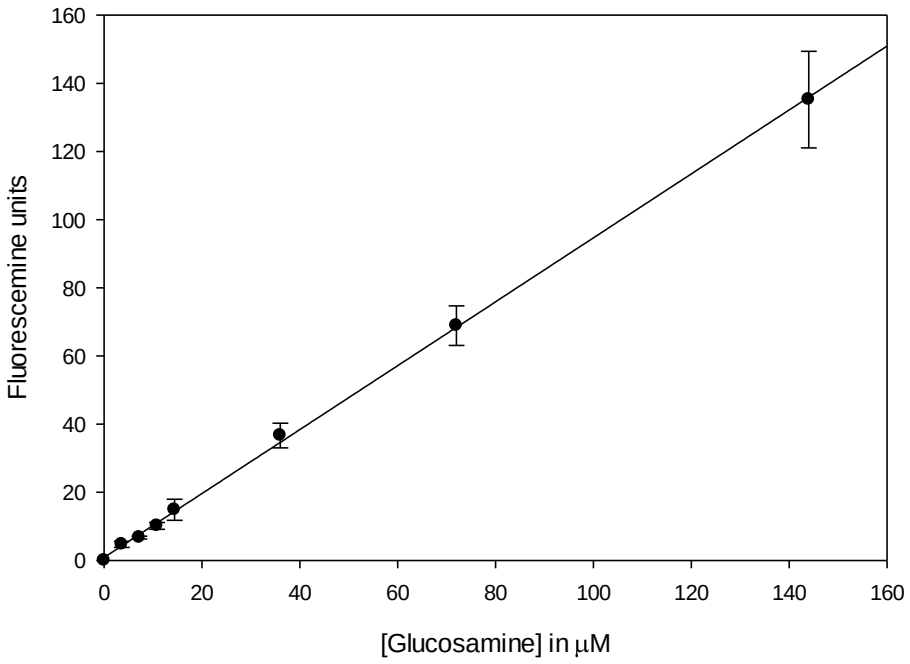


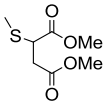
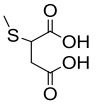
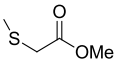
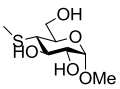
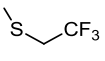
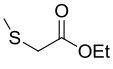
Fig. 3.10: Glucosamine standard curve obtained by fitting a linear equation (**y = mx + x**) through the data. Each point represents the amount of the fluorescence units observed for a specific concentration of glucosamine. Each point was done in triplicate with the error bars representing the standard deviation.

3.2.5.2 Evaluation of MshB deacetylation activity

The specific activity of MshB with a variety of substrate analogues has been reported to be between 5–66% relative to its native substrate, GlcNAc-Ins [18, 31, 41]. However, in the case of some alternative substrates (2,4DNP-α-GlcNAc and α-GlcNAc-SPh) for which MshB does show relatively high deacetylation activity, solubility under the assay conditions is a significant problem [31, 41]. In this study, the deacetylase activity of MshB was evaluated against compounds containing the GlcNAc moiety as shown in Table 3.2 including GlcNAc with anticipation to identify a compound(s) that can be potentially used as alternative substrates during inhibition studies. The compounds tested include GlcNAc linked with three types of glycosidic linkages (*O*-, *S*- and *N*-) and having different anomeric configurations (α- and β-). All compounds were tested at a final concentration of 5 mM with 1 µM of enzyme over a

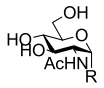
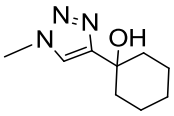
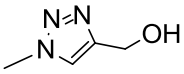
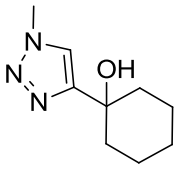
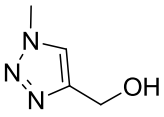
period of 1 h. Since α-GlcNAc-Ins was not available in this study, the specific deacetylation activity of MshB was related to its activity towards GlcNAc, as it one of the most used alternative substrates (MshB has been found to be 87 fold less active with GlcNAc as substrate in relation to its natural substrate, α-GlcNAc-Ins [18]). The results of the activity assays are summarized in Figure 3.11.

Table 3.2: Compounds synthesised as potential alternative substrates of MshB and/or BshB

Type of Glycoside	Compound		Type of synthesis	Described in chapter
O-glycoside	**1**		Chemical	3
	2		Chemical	3
S-glycoside	**3**		Enzymatic	3
	4		Chemical	3
	5		Enzymatic	2
	6		Chemical	3
S-glycoside	**7**		Chemical	2
	8		Enzymatic	2
	9		Enzymatic	2

Continued on the following page

Table 3.2 (continued)

Type of Glycoside	Compound		Type of synthesis	Chapter
N-glycoside	**10**	 (β-linkage)	Chemical	3
	11	 (β-linkage)	Chemical	3
	12	 (α-linkage)	Enzymatic	2
	13	 (α-linkage)	Chemical	3
	14	 (α-linkage)	Chemical	3
	15	 (β-linkage)	Purchased	Carbosynth

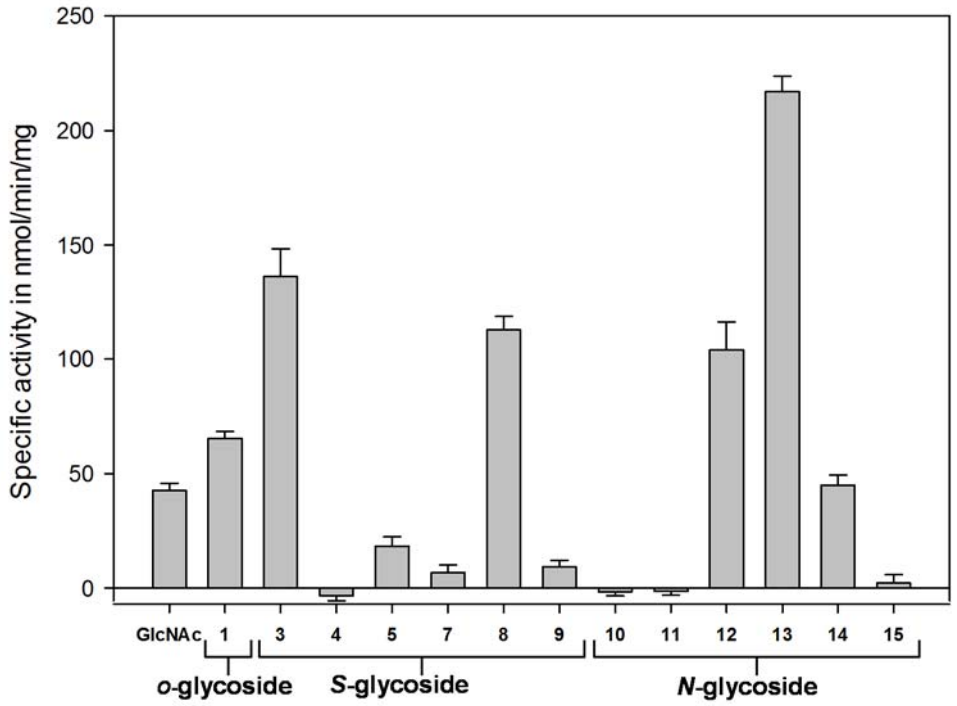


Fig. 3.11: Specific deacetylase activity of MshB determined with various potential alternative substrates. The bars represent the average specific activity of the different compounds with error bars representing standard deviation. Activity assays were performed using 5 mM of the indicated compound and 1 µM MshB, in duplicate. Compounds are identified by the numbers as listed in Table **3.2**. GlcNAc was used as reference compound for the specific deacetylase activity of MshB.

Using the FSA assay, the specific deacetylase activity of MshB with GlcNAc (the chosen reference compound) was found to be 42.6±3.1 nmol/min/mg. Its α-methyl glycoside (**1**) was found to be a better substrate, with a specific activity of 65.2±3.2 nmol/min/mg (a 1.53-fold increase). The BshB substrate α-GlcNAc-Mal (**2**), which was prepared by chemical synthesis, was not tested for MshB activity.

Amongst the *S*-glycosides (compounds **3**-**5**, **7**-**9**) no MshB deacetylase activity was observed including α-GlcNAc-SMal (**4**), the thioglycoside analogue of the BshB natural substrate, α-GlcNAc-Mal. However, MshB showed the most activity towards its methyl ester (α-GlcNAc-SMal$(OMe)_2$) (**3**), with a specific activity of 136±12.3 nmol/min/mg. The MshB activity decreased towards the other *S*-glycosides **5, 7, 8**, and **9** in relation to compounds 3, with activities of 18.3±4.12, 6.80±3.31 113±6.0 and 9.24±2.91 nmol/min/mg, respectively (Figure 3.11).

MshB activity towards the GlcNAc triazoles and azide (*N*-glycoside) was only observed for compounds with an α-anomeric configuration. Of these triazole **13**, which has a hydroxylated cyclohexane ring reminiscent of the inositol moiety of the MshB native substrate GlcNAc-Ins, showed the most deacetylase activity (217±6.7 nmol/min/mg), 5.09-fold more compared to GlcNAc (Figure 3.11). It was followed by α-GlcNAc-N_3 (**12**) and the triazole **14**, with specific activities of 104±12.5 nmol/min/mg and 44±4.4 nmol/min/mg, respectively. No deacetylase activity was observed with compounds having the β-glycosidic linkages (compounds **10**, **11** and **15**) demonstrating that MshB has a strong preference for compounds with the glycosidic bond in the α-anomeric configuration.

To determine if the observed activity was not due to the interaction of FSA with the tested compounds, the assay was repeated with compounds **1**, **3**, **5**, **8**, **9** and **12**–**15**) in the absence of MshB. Although low levels of background fluorescence were observed in the case of some compounds, these levels are not sufficient to significantly influence the activity rates observed (Figure 3.12).

Taken together, the results show that MshB clearly prefers the compounds with α-anomeric glycosidic bond over the β ones. The type of the glycosidic bond apparently does not influence or affect the activity, as MshB activity was observed with compounds with all three different types of the glycosidic bonds. However, the type of the glycosidic bond is an important consideration during the development of inhibitor compounds, as it has been found that S-glycosidic bonds are more resistant to hydrolysis than their *O*-glycoside counterparts [55]. In contrast to the lack of effect of the glycoside linkage, the structure of the moiety attached to GlcNAc clearly impacts on the activity of MshB. This corresponds with the results of the docking studies reported by Huang *et al*. that showed that the moiety attached to the GlcNAc influences the binding of the substrate at the active site, thereby promoting the activity of MshB [42].

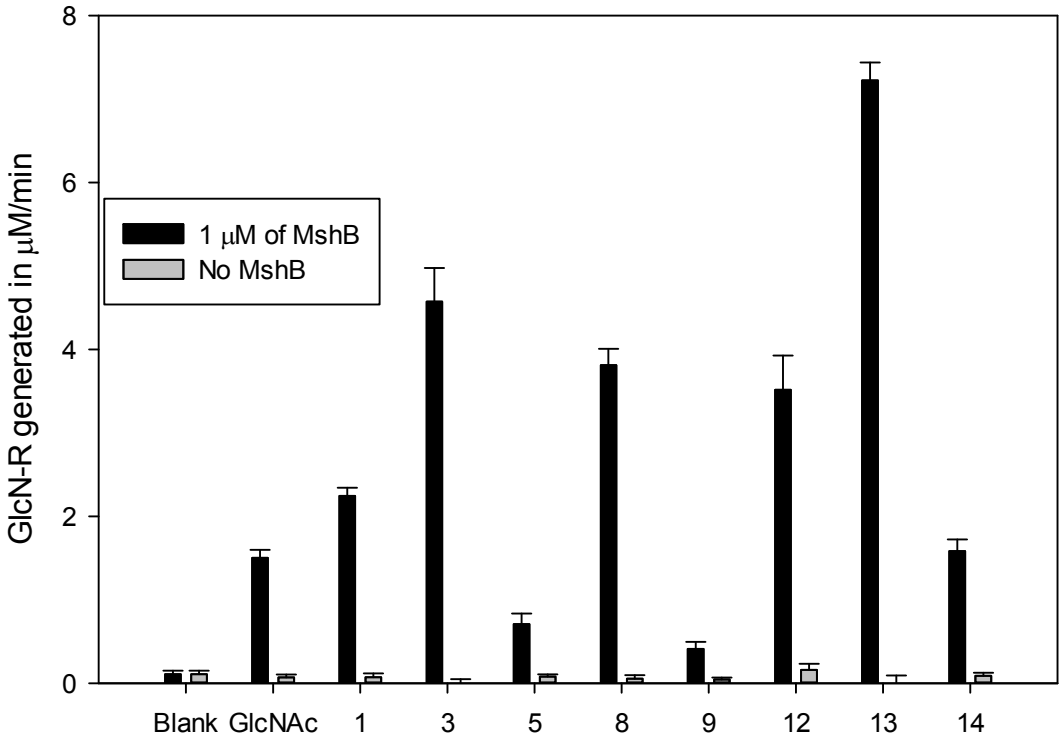


Fig. 3.12: Background fluorescence due to interaction of fluorescamine with the various potential alternative substrates. The bars represent average of the free amino sugar generated for the different compounds with error bars representing standard deviation. Activity assays were performed using 5 mM of the indicated compound and in present and absence of MshB, in duplicate. Compounds are identified by the numbers as listed in Table **3.2**. Blanks were used as reference compound for the background fluorescence activity.

3.2.5.3 Preliminary kinetic study of MshB activity towards alternative substrate candidates

Next we set out to determine the kinetic parameters of MshB with α-GlcNAc-triazole compound **13** and α-GlcNAc-N_3 **12** to establish how these compare to the values previously obtained for the enzyme's native substrate, GlcNAc-Ins. The thioglycosides **3** and **8** that also showed high levels of activity as MshB substrate were not included in these tests due to the limited availability of compounds. The kinetic studies were done with 1 µM of enzyme and substrate concentrations ranging from 0.1 mM to 5 mM in a time course over 50 minutes for each concentration tested using the FSA assay.

MshB exists as a dimer at normal ionic strength, while at low ionic strength two of these dimers combine to form tetrameric structures [18]. In addition, it also consists of a polar binding pocket distal from the GlcNAc binding site [18, 30], hence its ability to accommodate the inositol moiety of GlcNAc-Ins and likely the aglycone moieties of compounds **12** and **13**. During the kinetic study the data obtained for compounds **12** and **13** generated sigmoidal curves in both cases. This suggests the cooperative binding of the compounds by MshB. As such the kinetic parameters for both compounds were obtained by fitting the single substrate Hill equation **($v=V_{max}\times S^n/(K_s^n+S^n)$**, where n = Hill number**)** to the data(Figure 3.13). The kinetic parameters of compound **12** were established to be as follows: n = 2.1, K_S = 1.62 ± 0.104 mM, k_{cat} of 2.29 ± 0.086 nmoles/min and k_{cat}/K_S of 1.41 ± 0.105 $M^{-1}min^{-1}$, while that of compound **13** were as follows: n = 2.4, K_S = 1.74 ± 0.064 mM, k_{cat} of 8.70 ± 0.191 nmoles/min and k_{cat}/K_S of 5.03 ± 0.217 $M^{-1}min^{-1}$ (Figure 3.12). The K_S values observed for these compounds were 5-fold higher in relation the natural substrate (GlcNAc-Ins, K_M = 340 ± 80 µM as reported by Newton *et al.* [18]. However, it was much lower compared to the K_M obtained for GlcNAc (K_M 38 ± 4 mM) for *M. smegmatis* MshB by Huang *et al.* [43]. This suggests that both compounds (**12** and **13**) are far better alternative substrates for MshB than GlcNAc. In addition, the finding that the Hill numbers are very close to two would agree with the previous finding that MshB exists as a dimer. However, we were concerned that the observed sigmoidal activity profiles might be an artefact of the FSA assay, as no activity was observed for compound **13** at low concentrations (below 100 µM), even after several repetitions of the reaction. This suggested that the assay used might not be sensitive enough to report activity at low substrate concentrations, leading to its sigmoidal shape. This potential issue with the FSA assay's sensitivity was addressed in the work as described in chapter 4.

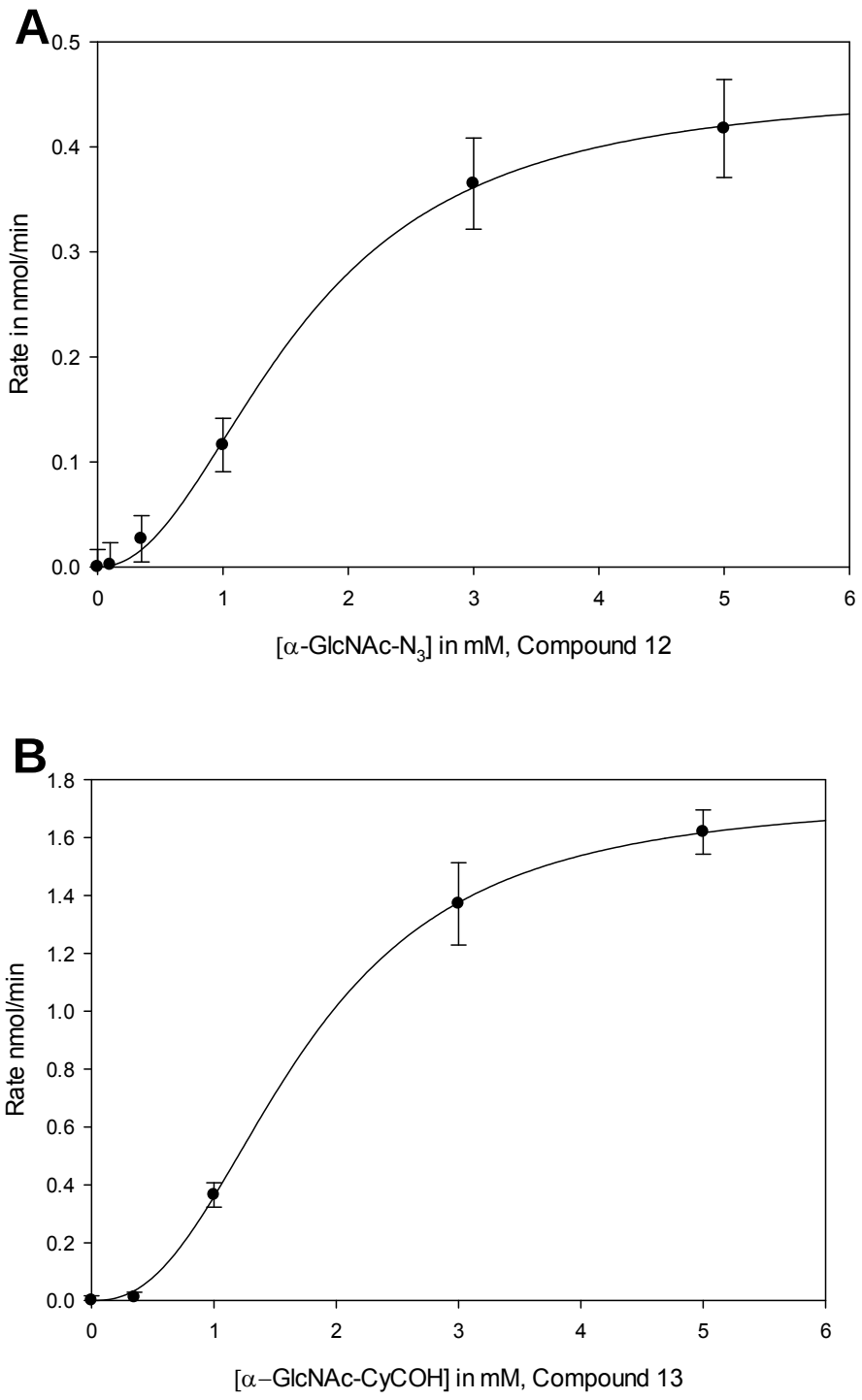


Fig. 3.13: Activity profiles for MshB with α-GlcNAc-N_3 **12** (**Panel A**) and α-GlcNAc-triazole **13** (**Panel B**) The data represent the average rate of MshB with the different compounds at various concentration with error bars representing standard deviation. Activity assays were performed using a range of concentrations (0–5 mM) of the different compounds, in duplicate. A curve using the single substrate Hill kinetic equation **($v=V_{max}\times S^n/(K_M^n+S^n)$)** was fitted through the data.

3.2.6 Inhibition of MshB activity by substrate analogues

Several compounds have been synthesised and tested as inhibitory compounds against MshB activity [30, 31]. However, they were found to inhibit the activity of the enzyme only at high concentrations, with IC_{50} values of between 2 and 7 µM. Consequently the need remains to find inhibitors with higher potency.

Gammon and co-workers recently synthesized several potential MshB inhibitors (data not published) based on the naphthoquinones scaffold explored previously by the same group [31]. Previously, Gammon and co-workers in a preliminary study found that naphthoquinones with 2 to 5 carbons as spacer are better inhibitors compounds. As such they chemically synthesised plumbagin derivatives alone and plumbagin tethered derivate with 2 to 5 methylene carbons spacer linked to phenyl-2-deoxy-2-amino-1 thio-α-D-glucopyranoside through amide bond (shown in Figure 3.14) as potential inhibitory compounds. We therefore set out to test the inhibition potency of these compounds toward MshB activity. For these tests, we used the continuous MshB assay that was previously developed in our group (see Chapter 4) [41]. This assay uses α-GlcNAc-S-DNP as substrate; after deacetylation, the substrate undergoes a rearrangement reaction that reveals a free thiol group that can be tracked using 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) also knowns as Ellman's Reagent. The use of FSA assay for the inhibition study was limited by two factors; lack of a good compound to use as alternative substrate and chances of the fluorescamine interaction with the free amino group within the structure of the compounds (Figure 3.14). The use of the NDA assay was not feasible because during the deacetylation activity characterisation of MshB NDA assay was also tested as an alternative assay, but yielded no results, that is, no activity could be detected.

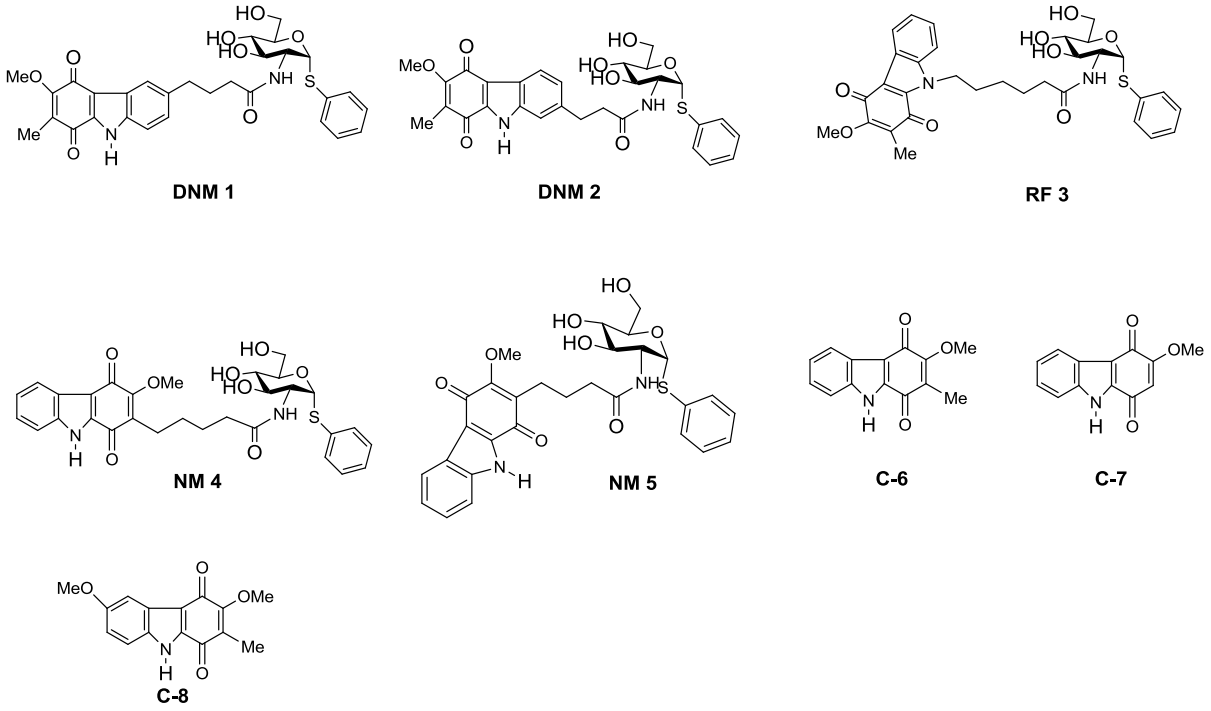


Fig. 3.14: Structures of the putative MshB inhibitors synthesized by the Gammon group (UCT).

Due to the limitations of the DTNB-based assay which will be discussed in chapter 4, section 4.2.5, the inhibition studies were done at two different inhibitor concentrations, i.e. 75 µM and 150 µM, using a substrate concentration of 150 µM. The observed activity in each case was normalized relative to a control reaction containing only DMSO which was set at 100% (Figure 3.15). Very few compounds showed more than 20% inhibition under these conditions. Only two compounds (RF 3 and NM 4) showed the most inhibitory activity and had the longest methylene carbons spacer between plumbagin tethered and phenyl-2-deoxy-2-amino-1 thio-α-D-glucopyranoside in relation to other compounds (Figure 3.14). Most inhibition activity for both compounds was observed with the highest concentration (150 µM) i.e. RF3 showed 71%, while NM4 showed 51% inhibition activity. The inhibition activity at 75 µM of both compounds (68% and 44% for RF3 and NM4, respectively) was 6 fold less to the one at 150 µM indicating that neither compound was an effective inhibitor compounds. This suggests that the maximum inhibition levels under the assay conditions have been reached. Interestingly, of the other inhibitory compounds, compound DNM2 was found to actually stimulate MshB activity, rather than inhibit it.

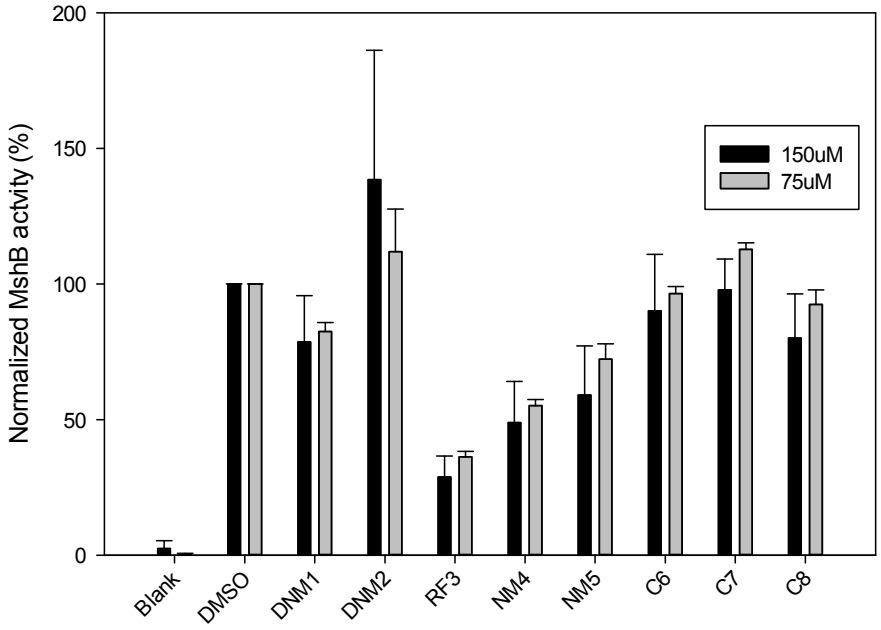


Fig. 3.15: Inhibition of MshB (3 μM) activity by the putative inhibitor compounds at either 75 μM (grey bars) or 150 μM (black bars) using 150 μM α-GlcNAc-S-DNP as substrate. The bars represent MshB deacetylation activity with α-GlcNAc-S-DNP as the alternative substrate in the presence of inhibitory compounds, with error bars representing standard deviation. The activity of the enzyme was normalised to a sample containing only DMSO, i.e. with no inhibitory compound present.

3.2.7 Characterisation of BshB

Although at the start of this study our intention was to perform all the tests described above for MshB with BshB (especially since some of the compounds prepared chemo-enzymatically are structurally very close analogues of this enzyme's native substrate) we were unable to obtain active enzyme with which to perform these tests. BshB was expressed and purified twice at different times and the purified enzyme tested for deacetylase activity using GlcNAc or compounds **2**, **3** and **4** as substrates. Both the FSA and NDA assays were used for these tests. In all cases no BshB activity could be detected. Previous studies have indicated that the loss of the enzyme's metal cofactor is a likely cause for the loss of enzyme activity. However, even in cases where the enzyme was subsequently treated with Zn^{2+} (its preferred cofactor) no activity could be observed. We have therefore initiated a collaboration with the group that first characterized BshB activity to allow us to continue this part of the study. However, at the time of writing this had not been achieved as yet.

3.3 Summary and Conclusion

The application of α-*N*-acetyl-thioglycosaminoligases as biocatalyst using the same strategy as in a previous study (see Chapter 2) allowed the generation of α-GlcNAc-S-Mal $(OMe)_2$ and through chemical modification of α-GlcNAc thioglycosides (α-GlcNAc-SMal$(OMe)_2$ and α-GlcNAc-SCH_2CO_2Me) obtained by these biocatalysis BshB analogues, α-GlcNAc-SCH_2CO_2H and α-GlcNAc-SMal were synthesised. In addition, α-GlcNAc-N_3 also generated from these biocatalysts permitted the chemical synthesis of GlcNAc-triazoles through "Click chemistry". This indicates that the α-*N*-acetyl-thioglycosaminoligases are useful for synthesis of α-GlcNAc thioglycosides and through careful selection of thio-molecules as acceptor and reaction conditions many more α-GlcNAc thioglycosides of biomedical interest can be generated.

Due to the lack of generating the excepted reaction product (GlcN-R) of the various GlcNAc-based glycosides (Table 4.2) tested as alternative substrate for MshB, glucosamine was used to generate the standard curve for the conversion of fluorescence units into concentration of GlcN-R producing the reaction. Despite this small problem the characterisation of MshB with several α-GlcNAc-based glycosides (Table 4.2) showed that the type of glycosidic bond does not influence the activity of MshB, while the anomeric configuration and GlcNAc moiety does. Looking at Figure 3.11, compound **3**, **8, 12** and **13** can be viewed as potential substrate for MshB, as they were way more active than GlcNAc. The preliminary kinetic study with compounds **12** and **13** also showed that these compounds are better alternative substrates than GlcNAc. Observing the data from the specific activity to the inhibition study, the lack of generating the standard reaction product and the lack of a good continuous assay to characterise MshB are the major problems. In addition, discontinuous assays are time consuming and they bring about human error which can really affect the final data leading to wrong conclusion. The development of a high throughput continuous assay will open many doors for characterisation of MshB and BshB with the generated α-GlcNAc-based glycosides.

3.4 Materials and methods

All the chemicals used were purchased from Sigma-Aldrich unless stated otherwise except, GlcNAc which was brought from Carbosynth. 2-(S)-(3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-**D**-glucopyranosyl)-butan-1,4-dioic acid dimethyl ester was obtained

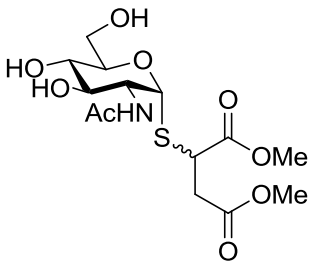
3.4 Materials and methods

All the chemicals used were purchased from Sigma-Aldrich unless stated otherwise except, GlcNAc which was brought from Carbosynth. 2-(S)-(3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-glucopyranosyl)-butan-1,4-dioic acid dimethyl ester was obtained as a present from Chris Hamilton group. The assays were done using Greiner Bio-One polystyrene flat-bottomed 96-well plates, specifically clear plates for the DTNB assay and black plates for FSA assay. Varioskan multimode reader (Thermo Scientific) was used to measure the fluorometric and spectrophotometric readings. Enzyme expression and purification was done based on Newton et al for MshB [18] and based on Fang et al for BshB [46]. All cultures were grown until the optical density at 600 nm (OD_{600}) reached 0.600, followed by induction with 0.10 mM isopropyl-1-thio-β-D-galactropyranoside (IPTG) for MshB and 5.8 mM of lactose for BshB followed by incubation of the cultures overnight at 22°C and 15°C, respectively. This was followed by the harvesting of cells by centrifugation at 17000×*g* for 20 minutes at 10°C. The pelleted cells were stored at -20°C until use. Both enzymes were subject to disruption by sonication external cooling within their respective buffer before purification. The enzymes were purified with automated purification program on ÄKTA*prime* system (Amersham Bioscience) using the previously published described methods after the removal of the cell bris through centrifugation [18, 46]. SDS-PAGEs (12%) were used to check the purity of the proteins, while the Bradford method was used to determine the protein concentration.

3.4.1 Generation of GlcNAc thioglycosides

3.4.1.1 Enzymatic synthesis

Dimethyl *S*-(2-acetamido-2-deoxy-α-D-glucopyranosyl) 2-thiosuccinate (3): The

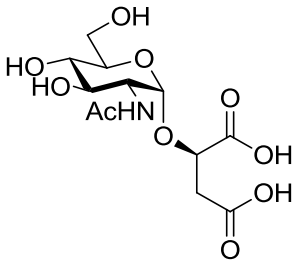


same reaction conditions described in chapter 2 were for the enzymatic synthesis of **3**. ^{1}H NMR (600 MHz, d_2o) δ 5.73 (d, *J* = 5.5 Hz, 1H), 5.62 (d, *J* = 5.4 Hz, 1H), 4.09 (td, *J* = 11.4, 5.4 Hz, 1H), 3.98 (ddt, *J* = 13.4, 6.8, 5.1 Hz, 2H), 3.89 – 3.80 (m, 2H), 3.79 (d, *J* = 0.8 Hz, 3H), 3.73 (d, *J* = 3.4 Hz, 3H), 3.65 – 3.58 (m, 1H), 3.56 – 3.50 (m, 1H), 3.10 (ddd, *J* = 33.2, 17.1, 8.5 Hz, 1H), 2.95 (ddd, *J* = 37.3, 17.1, 6.5 Hz, 1H), 2.05 (s, 3H). ^{13}C NMR (150 MHz, d_2o) δ 174.93 (s), 174.83 (s), 174.57 (s), 174.43 (s), 173.72 (s), 84.45 (s), 83.50 (s), 73.78 (s), 73.68 (s), 71.31 (s), 71.25 (s), 70.59 (s), 70.48 (s), 60.83 (s), 60.67 (s), 54.49 (s), 54.36 (s),

54.00 (s), 53.98 (s), 53.23 (s), 53.16 (s), 42.78 (s), 40.82 (s), 37.13 (s), 36.26 (s), 22.36 (s). ESI-MS (*m/z*): $[M+H]^+$ calcd. for $C_{14}H_{24}NO_9S$ 382.1167; found 382.1170.

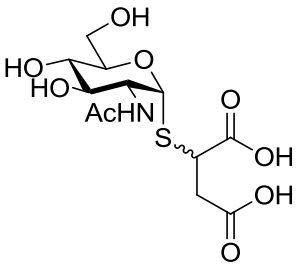
3.4.1.2 Chemical synthesis

2-(*R*)-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-oxy-succinic acid (2).



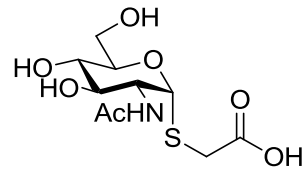
Previously described methods by Sharma *et al.* were used to synthesise α-GlcNAc-O-Mal [13]: To 2-(S)-(3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-glucopyranosyl)-butan-1,4-dioic acid dimethyl ester group (0.123 g, 0.19 mmol) in dry ethyl acetate at 0°C was added 555 µL of acetic anhydride and 56 mg of 10% Pd/C. This was followed by purging the reaction with nitrogen followed by addition of 285 µL triethylsilane drop wise over an hour. The reaction was allowed to warm up to room temperature and stirred overnight. This was followed by filtering the reaction mixture through a pad of Celite and washed with ethyl acetate. After the removal of solvent the mixture was co-evaporated with toluene three times. To the crude in dry methanol at 0°C was added 1 mL of 1M NaOCH3 and stirred for 2hrs. Dowex-H^+ resin (200mg) was used to quench the reaction. After filtration of the resin the solvent was evaporated with the crude subjected to flash chromatography (10:1 to 1:1, ethyl acetate: methanol) to produced 6.55 mg of α-GlcNAc-O-Mal as white powder, with 10% yield over two step. The NMR analyses gave spectra identical to those reported by Sharma *et al* [13].

***S*-(2-acetamido-2-deoxy-α-D-glucopyranosyl) 2-thiosuccinic acid (4)**:α-GlcNAc-SMal was generated from compound 3by removing the methyl group using the method of White *et al.* and Corey *et al.* [52, 53]. Dimethyl ester **3** (11.18 mg, 0.0293 mmol) was dissolved in THF: H_2O (3:1) mixture at 0°C. This was followed by addition of lithium hydroxide (1.58 mg, 0.065 mmol) and stirred at room temperature until the starting material disappeared. The reaction mixture was concentrated under reduced pressure to remove all the solvents. The resulting residue was purified by flash column chromatography (61:33:6 DCM:methanol:water) to give a white powder (6.10 mg, 59%). ^{1}H NMR (600 MHz, D_2O) δ 5.53 (t, *J* = 4.9 Hz, 1H), 4.12 (dt, *J* = 10.5, 5.2 Hz, 1H), 4.09 – 4.02 (m, 1H), 3.92 – 3.80 (m, 2H), 3.72 – 3.60 (m, 2H), 3.50 (dd, *J* =

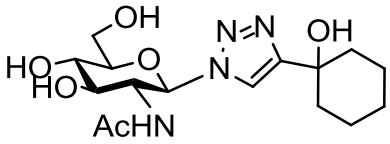


20.2, 10.1 Hz, 1H), 2.76 (ddd, *J* = 15.1, 6.2, 2.4 Hz, 1H), 2.48 (ddd, *J* = 36.0, 15.3, 9.5 Hz, 1H), 2.06 (s, 3H). ^{13}C NMR (150 MHz, D_2O) δ 179.14 (s), 179.07 (s), 179.02 (s), 179.01 (s), 174.35 (s), 135.22 (s), 83.99 (s), 82.53 (s), 72.56 (s), 72.52 (s), 71.09 (s), 71.00 (s), 70.23 (s), 70.08 (s), 60.41 (s), 60.19 (s), 53.75 (s), 53.61 (s), 48.17 (s), 46.04 (s), 41.24 (s), 40.48 (s), 21.87 (s), 21.86 (s). ESI-MS (*m/z*): $[M+H]^+$ calcd. for $C_{12}H_{20}NO_9S$ 354.0854; found 354.0860

***S*-(2-acetamido-2-deoxy-α-D-glucopyranosyl) 2-thioacetic acid (6)**: α-GlcNAc-S-CH_2-COOH was synthesised using the same methods used for α-GlcNAc-SMal above from compound **5** which was enzymatically synthesised in Chapter 2. Compound **5** (12.2 mg, 0.039mmol) and lithium hydroxide (1.74 mg, 0.072 mmol) gave 3.54 g of α-GlcNAc-S-CH_2-COOH as white powder (31% yield). ^{1}H NMR (400 MHz, D_2O) δ 5.43 (d, *J* = 5.3 Hz, 1H), 4.11 (dd, *J* = 11.0, 5.3 Hz, 1H), 4.073 – 4.073(m, 1H), 3.87 – 3.76 (m, 2H), 3.72 (dd, *J* = 10.9, 8.9 Hz, 1H), 3.49 (dd, *J* = 12.1, 6.9 Hz, 1H), 3.35 – 3.18 (m, 2H), 2.04 (s, 3H). ^{13}C NMR (100 MHz, D_2O) δ 178.05 (s), 175.10 (s), 84.37 (s), 73.13 (s), 71.60 (s), 70.89 (s), 61.01 (s), 54.41 (s), 36.05 (s), 22.53 (s).

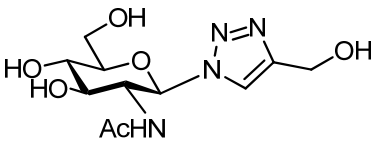


***N*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-4-(1-hydroxycyclohexyl)-1H-1,2,3-triazole (10).** Previously established synthetic method by Rostovtsev *et al.* was used for the synthesis of compound **10** [48]. To a solution of β-GlcNAc-N_3 (50 mg, 0.20 mmol) and 1-ethynyl-1-cyclohexanol (65 mg, 0.52 mmol, 67 µL) in 1.7 mL mixture of water/tert-butyl alcohol/ACN /DMF (1:1:2:1) stirring at room temperature was added 186 µL of sodium ascorbate (0.26 mmol, 1.4 M solution freshly prepared in water). This was followed by addition of 142 µL copper (II) sulfate pentahydrate (4.7 mg dissolved in water, 0.01882 mmol). The mixture was stirred overnight at room temperature. TLC was used to check for the complete consumption of the β-GlcNAc-N_3. The reaction mixture was concentrated under reduced pressure to remove all the solvents. The resulting residue was purified by flash column chromatography (7:2:1 ethyl acetate:methanol:water) to give the



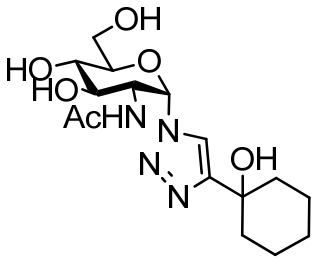
1H), 3.96 (dd, *J* = 12.5, 2.0 Hz, 1H), 3.91 – 3.67 (m, 4H), 2.14 (m, 2H), 1.87 – 1.76 (m, 5H), 1.70 (m, 2H), 1.53 – 1.28 (m, 4H). ^{13}C NMR (100 MHz, D_2O) δ 174.57 (s), 122.02 (s), 87.05 (s), 79.56 (s), 74.14 (s), 71.39 (s), 70.28 (s), 69.94 (s), 61.06 (s), 56.05 (s), 37.61 (s), 37.58 (s), 25.37 (s), 22.67 (s), 22.63 (s), 22.19 (s). ESI-MS (*m/z*): $[M+Na]^+$ calcd. for $C_{14}H_{26}N_4O_6Na$ 393.1744; found 393.1754

***N*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-4-hydroxymethyl-1H-1,2,3-triazole**



(11): The same procedure as above was used to generate the title compound: β-GlcNAc-N_3 (50 mg, 0.20 mmol) and propargyl alcohol (29 mg, 0.52 mmol, 30 µL). The resulting residue was purified by flash column chromatography (7:2:1 ethyl acetate:methanol:water) to give the product as a whitish powder (38.73 mg, 64%). ^{1}H NMR (400 MHz, D_2O) δ 8.20 (s, 1H), 5.87 (d, *J* = 9.7 Hz, 1H), 4.74 (s, 2H), 4.28 (t, *J* = 9.9 Hz, 1H), 3.96 (dd, *J* = 12.5, 2.0 Hz, 1H), 3.89 – 3.81 (m, 2H), 3.81 – 3.75 (m, 1H), 3.75 – 3.68 (m, 1H), 1.83 (s, 3H). ^{13}C NMR (75 MHz, d_2o) δ 174.82 (s), 147.69 (s), 123.33 (s), 86.98 (s), 79.53 (s), 74.12 (s), 69.91 (s), 61.05 (s), 55.98 (s), 55.16 (s), 22.23 (s). ESI-MS (*m/z*): $[M+Na]^+$ calcd. for $C_{11}H_{24}N_4O_6Na$ 325.1118; found 325.1129.

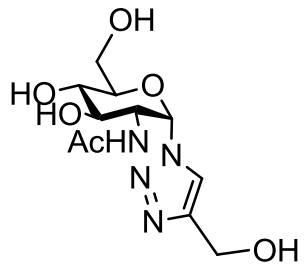
***N*-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-4-(1-hydroxycyclohexyl)-1H-1,2,3-triazole (13).**



The previously established synthetic method of Machida *et al* was used for the synthesis of compound **13** [54]. To a solution water of α-GlcNAc-N_3 (19.52 mg, 0.08 mmol, 40mM) and copper(II) sulfate pentahydrate (0.016 mmol, 4.0 mg) stirring on ice was added sodium ascorbate (0.16 mmol, 32 mg) and 600 µL of TBTA (8.5 mg, 0.016 mmol) dissolved in DMF: tert-butyl alcohol (1:1). This was followed by addition of 1-ethynyl-1-cyclohexanol (9.93m g, 0.08 mmol, 10 µL). The reaction was stirred on ice for 5 hrs followed by stir at room temperature overnight. TLC was used to check for the complete consumption of the α-GlcNAc-N_3. After complete consumption of the α-GlcNAc-N_3 the reaction mixture was concentrated under high pressure to remove all the solvents. The final product was a white powder (11.17mg, 38%). ^{1}H NMR (400 MHz,

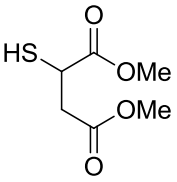
the reaction mixture was concentrated under high pressure to remove all the solvents. The final product was a white powder (11.17mg, 38%). ^{1}H NMR (400 MHz, d_2o) δ 8.07 (s, 1H), 6.36 (d, *J* = 5.8 Hz, 1H), 4.58 – 4.50 (m, 1H), 4.42 (dd, *J* = 10.8, 5.8 Hz, 1H), 3.82 – 3.63 (m, 4H), 2.17 – 2.07 (m, 2H), 1.90 – 1.79 (m, 5H), 1.76 – 1.65 (m, 2H), 1.57 – 1.34 (m, 4H). ^{13}C NMR (101 MHz, d_2o) δ 175.37 (s), 134.14 (s), 124.92 (s), 83.52 (s), 75.77 (s), 71.39 (s), 70.53 (s), 70.27 (s), 60.93 (s), 53.70 (s), 37.64 (s), 37.55 (s), 25.36 (s), 22.59 (s), 22.55 (s), 22.07 (s).). ESI-MS (*m/z*): $[M+Na]^+$ calcd. for $C_{14}H_{27}N_4O_6Na$ 371.1924; found 371.1933

***N*-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-4-hydroxymethyl-1H-1,2,3-triazole**



(14): The same procedure as above was used for the generation of compound **14**: α-GlcNAc-N_3 (14.22 mg, 0.06 mmol), copper(II) sulfate pentahydrate (0.012 mmol, 3.0 mg), sodium ascorbate (0.12 mmol, 24 mg), TBTA (6.37 mg, 0.012 mmol) and propargyl alcohol (3.36 mg, 0.06 mmol, 3.5 µL). The resulting residue was purified by flash column chromatography (7:2:1 ethyl acetate: methanol: water) to give a whitish powder (7.73 mg, 43%). ^{1}H NMR (400 MHz, D_2O) δ 8.09 (s, 1H), 6.37 (d, *J* = 5.8 Hz, 1H), 4.77 – 4.75 (m, 2H), 4.60 – 4.52 (m, 1H), 4.46 (dd, *J* = 10.8, 5.8 Hz, 1H), 3.82 – 3.64 (m, 4H), 1.89 (s, 3H). ^{13}C NMR (100 MHz, D_2O) δ 175.42 (s), 147.22 (s), 126.29 (s), 83.61 (s), 75.68 (s), 71.52 (s), 70.56 (s), 60.93 (s), 55.07 (s), 53.56 (s), 22.10 (s). ESI-MS (*m/z*): $[M+Na]^+$ calcd. for $C_{11}H_{24}N_4O_6Na$ 325.1118; found 325.1119.

Dimethyl 2-mercaptosuccinate: The title compound was synthesised by shielding the carboxylic acid of mercaptosuccinic acid based on the methods by Kikionis *et al.* [56] . To a mixture of mercaptosuccinic acid (2.5 g, 0.016 mol) in methanol (15 mL) at 0°C was added acetyl chloride (149 µL) dropwise and left to reflux at 60°C for 6hrs. After completion the reaction mixture was concentrated under reduced pressure to remove all the solvents. The resulting liquid was purified by flash column chromatography (2:1 hexane: ethyl acetate) to give a clear stinking liquid (2.06 g, 72%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 3.77 (s, 3H), 3.70 (s, 3H), 3.02 (dd, *J* = 17.0, 8.9 Hz, 1H), 2.77 (dd, *J* = 17.0, 5.9 Hz, 1H), 2.22 (d, *J* = 9.6 Hz, 1H), 1.59 (s, 1H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 172.70 (s), 170.71 (s), 52.81 (s), 51.99 (s), 39.59 (s), 35.94 (s). ESI-MS (*m/z*): $[M+Na]^+$ calcd. for $C_6H_{16}O_5SNa$ 201.0192; found 201.0195



3.4.2 The generation of the Standard curve

The same experimental procedure for the FSA assay was used for the generation of the standard curve data. Different concentrations of glucosamine (0 – 1 mM) in the reaction buffer were added into 20% of 10 µL of Trichloroacetic acid (TCA) and subjected to centrifugation at 13000 xg for 10 minutes. After the centrifugation process 25 µL of the supernatant was added into a 96 well plate, wherein 75 µL of 1M borate at pH 9.0 was added. The increasing concentrations reaction mixtures of glucosamine were reacted with 30 µL of 10 mM FSA dissolved in ACN. The FSA was allowed to react with the increasing concentrations reaction mixtures of glucosamine at room temperature for 10 minutes. The increasing concentrations reaction mixtures of glucosamine with FSA were subsequently determined by fluorimetry (FSA excitation at 395 nm and emission at 485 nm).

3.4.3 Fluorescamine assay

The MshB alternative substrate assays were done in assay buffer (50 mM HEPES, 50 mM NaCl, 1 mM TCEP) with 1 µM of MshB (final concentration) in a 200 µL reaction mixture. The reactions were done in duplicate and were initiated by addition of MshB after equilibrating the reactions at 30^{o}C for 10 minutes. The reactions were carried out at 30^{o}C for 1h, with 30 µL aliquots removed at 0, 5, 10, 30 50 and 60 minutes, followed by quenching with 10 µL of 20% TCA followed by removal of the precipitated enzymes by centrifuging at 13000×g for 10 minutes. The supernatants (25 µL) were transferred to 96-well black plates, to which was added 75 µL of 1M borate buffer at pH 9.0. The free amino sugar product was detected by addition of 30 µL 10mM FSA dissolved in acetonitrile followed by incubation in the dark for 10 minutes. The adduct levels were subsequently determined by fluorimetry (FSA excitation at 395 nm and emission at 485 nm.

3.4.4 DTNB continuous assay

The DTNB assay was carried out in the same way as published by Lamprecht *et al,* except that 3 µM of MshB was used and the reactions were done in a total volume of 150 µL [41].

3.4.5 NDA assay

The NDA assay was carried out in the same way as published by FANG *et al.*, different concentration of BshB was used and the reaction was done in a total volume of 150 µL [46].

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Chapter 4:

Studies towards the development of a high-throughput continuous assay of the metallo-deacetylases, MshB and BshB

4.1 Introduction

MshB and BshB are metal-containing deactylase enzymes that catalyse the third reaction of MSH biosynthesis pathway [1-4] and second reaction of BSH biosynthesis pathway, respectively [5-8]. Both reactions produce acetate and a free amino-sugar: MshB generates α-GlcN-Ins from α-GlcNAc-Ins, while BshB produces α-GlcN-Mal from α-GlcNAc-Mal (Figure 4.1). As stated previously, full characterization of MshB and BshB as well as the discovery of selective inhibitors targeting these enzymes are hampered by two factors. The first is the lack of availability of the natural substrates (or alternative compounds that can be used as substrates), especially in the case of MshB. This is mostly due to the challenge associated with the generation of the α-glycosidic bond in these compounds; a solution towards this is described in Chapter 3. The second factor is the lack of a high-throughput continuous assay that can be used for activity characterization and inhibitor screening. This is because neither substrates nor the amino-sugars generated from these reactions are UV-active, which makes it impossible to follow these reactions spectrophotometrically. Consequently, it becomes necessary to either derivatise the product to allow it to be quantified in a discontinuous manner, or for the formation of one of the products to be coupled to secondary enzyme reactions that can be assayed in continuous fashion. This part of the study describes the efforts that were made to develop such an assay for MshB and BshB.

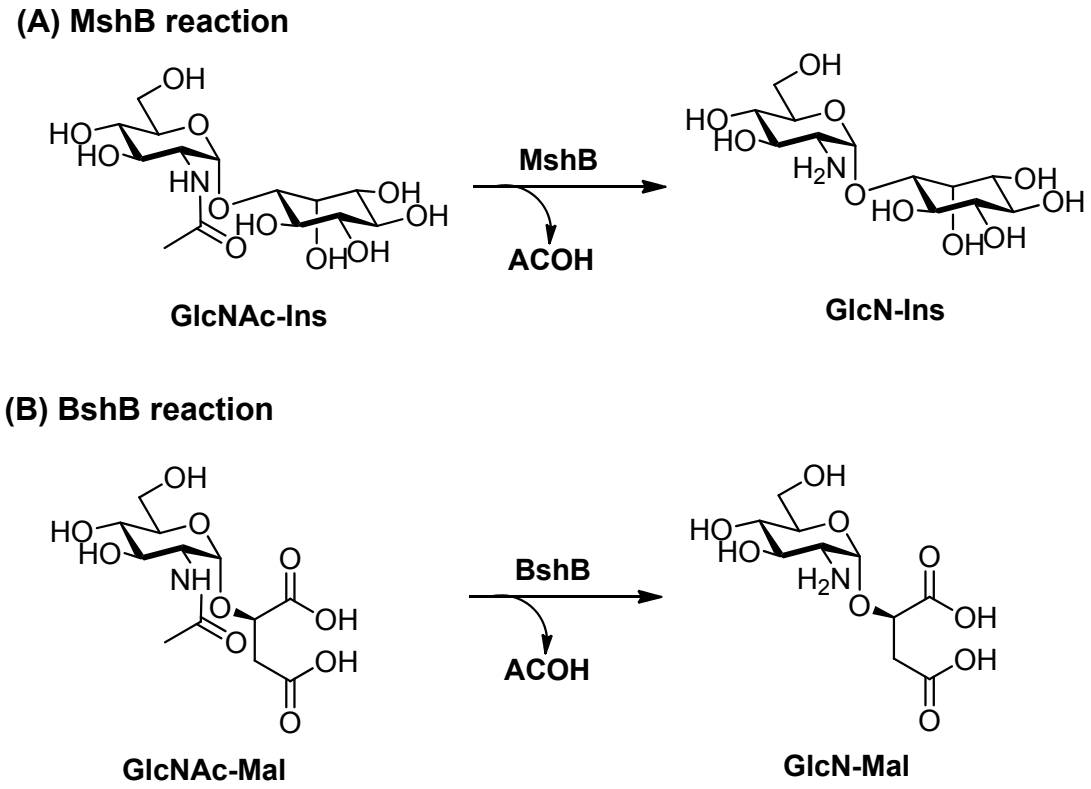

Fig. 4.1: The MshB (**A**) and BshB (**B**) reactions with their respective natural substrate and products

4.2 Assays currently utilised for determining metallodeacetylase activity, and their shortcomings

The majority of the assays that are currently used to characterise MshB and BshB activity are discontinuous assays that utilise derivatization agents such as 6-aminoquinolyl-*N*-hydroxysuccinimidly carbamate (AccQ-Fluor^{TM}) [3, 4], fluorescamine (FSA) [2] and naphthalene-2,3-dicarboxaldehyde (NDA) [7]. These derivatization agents react with the free amine group of the product generating a complex that is either spectrophotometrically or fluorometrically active. In addition, our group was involved in the development of a DTNB-based continuous assay for characterisation of MshB activity [9]. These various assays and their respective shortcomings are described below.

4.2.1 The AccQ-Fluor™ derivatization-based assay

AccQ-Fluor is a highly stable fluorescent reagent that reacts with primary and secondary amino group to produce the corresponding an *N*-6-aminoquinolyl-urea and *N*-hydroxysuccinimide as side product (Figure 4.2) [10]. It was synthesised by Cohen and Michaud in 1993 to overcome shortcomings of other fluorescent derivatization agents such as *o*-phthalaldehyde (OPA), 9-fluorenylmethylchloroformate etc [10]. These included lability, generation of numerous derivatives, and the inability to derivatise secondary amino groups. Amino groups derivatised with AccQ-Fluor can easily be analysed by High Performance Liquid Chromatography (HPLC) using reversed phase columns and fluorescence detection with excitation at 250 nm and emission at 395 nm. AccQ-Fluor has been found to work best under basic conditions, that is, it works well in solutions with a pH range from pH 8.3-10.0. The AccQ-Fluor derivatization-based assay is sold in kit-format by Waters Corporation.

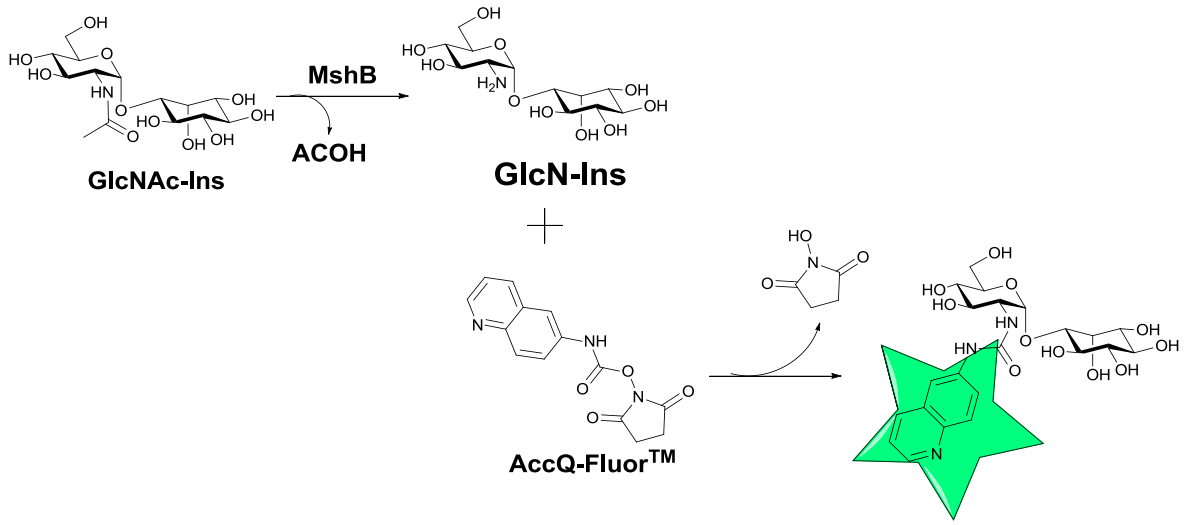

Fig. 4.2: The reaction of AccQ-Fluor with the free amino sugar of MshB reaction product.

The AccQ-Fluor kit has been used by Anderberg *et al.* in 1998 to investigate the formation of GlcN-Ins during MSH biosynthesis [11]. It was subsequently used in 2000 by Newton *et al*. in the determination of GlcNAc-Ins as the natural substrate of MshB and in the identification of GlcNAc-Ins as the key intermediate within the MSH biosynthesis pathway [3]. Since then it has been used by several people to characterise MshB activity [4, 9, 12]. In 2010 it was also applied by Parsonage *et al.*

in the discovery and characterisation of BshB involved the BSH biosynthesis pathway [13]. Apart from being a discontinuous assay, the use of AccQ-Fluor kit has its own shortcomings. It requires the use of HPLC to isolate and identify the derivatised product, which requires several hours for the analysis of multiple samples. In addition, the derivatization involves several steps that must occur before HPLC analysis, as required by the manufacture.

4.2.2 The FSA derivatization-based assay

FSA is a heterocyclic dione compound that is fluorescent once it reacts with a primary amine [14]. In 1972 Udenfriend *et al.* reported that FSA was synthesised by Weigele *et al*. as a substitute for fluorogenic ninhydrin reaction for the analysis of amino acids, primary amines and peptides [14]. FSA reacts with primary amines to form fluorophores that fluoresce when excited at 390 nm; the emission is at 475 mm. The best derivatization was found to occur at pH 9.0 [14, 15]. The first use of fluorescamine as a derivatizing reagent for MshB product formation was done by Huang *et al.* in 2011 (Figure 4.3) [2]. In this study the authors used GlcNAc as the substrate, and derivatised the GlcN product with FSA in 1M borate buffer at pH 9.0 followed by determination of the fluorescence in a 96 well plate using a plate reader.

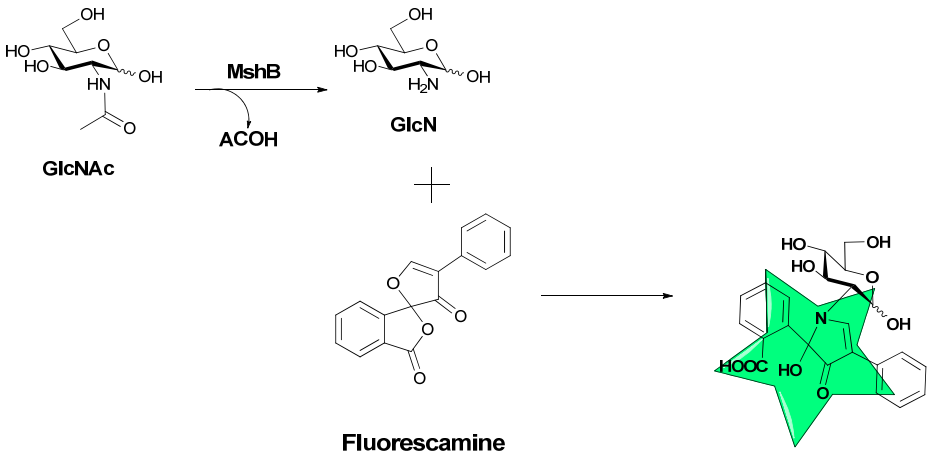

Fig. 4.3: MshB reaction through analysis of FSA-derivatization-based assay.

Apart from being a discontinuous assay, the FSA-derivatization-based assay requires generation of a standard curve using known standards of the expected reaction product to allow its quantification. With the MshB and BshB products this

can be very challenging due to the α-configuration of the glycoside that impedes access the free amine group on the 2-position.

4.2.3 The NDA derivatization-based assay

NDA is a fluorogenic derivatizing reagent that reacts with primary amines in the presence of a thiol or cyanide ion to produce an *N*-2-substituted-1-cyanobenz-[*f*]-isoindole, which is highly fluorescent (Figure 4.4) [16]. It was initially synthesised by Matuszewski and co-workers in 1986 as a replacement of OPA, a fluorogenic reagent which is very unstable at room temperature [17].

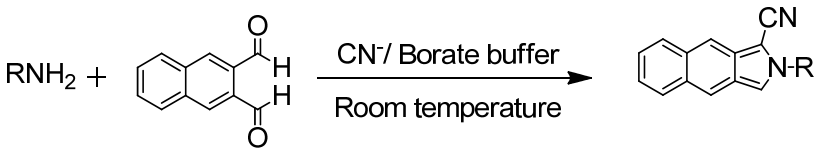

Fig. 4.4: The reaction of NDA with a primary amine in the presence of cyanide at room temperature [16].

Since then it has being used by many as the fluorogenic derivatising agent of choice for primary amines due to its improved stability and sensitivity compared to FSA and AccQ-Fluor [7, 14, 15]. Recently, NDA was used as the fluorogenic derivatising agent in the characterisation of BshB [7].

The major issue with the NDA assay is that it is also a discontinuous assay, and like FSA it requires the generation of a standard curve using known standards of the expected reaction product to allow its quantification. In addition, it also requires the use of a toxic molecule, namely potassium cyanide.

4.2.4 The DTNB-based continuous assay

In 2012 Lamprecht *et al.* developed a DTNB-based continuous assay for the characterisation of MshB activity [9]. This assay depends on an intramolecular S→N Smiles rearrangement that occurs after the deacetylation of 2,4DNP-α-*S*-GlcNAc (an alternative substrate) by MshB (Figure 4.5). The intramolecular S→N Smiles rearrangement takes place in three steps which is highly dependent on the nature of the substituent(s) of the aromatic group. The first step involves reaction of the amine with the nitrogen of the GlcN moiety generated after the deacetylation of 2,4DNP-α-

S-GlcNAc with carbon 1 of phenyl ring to form the spiro-Meisenheimer intermediate. This is followed by its deprotonation to form a nucleophilic spiro-Meisenheimer intermediate. The last step involves the bond breaking between carbon 1 and the sulphur atom generating a thiolate anion which is then protonated to form a thiol group. Once the thiol is generated DTNB reacts with it to form NTB while forming a disulphide bond with the thiol group of the reaction product. NTB is UV-active and can be detected at 412 nm as it is released.

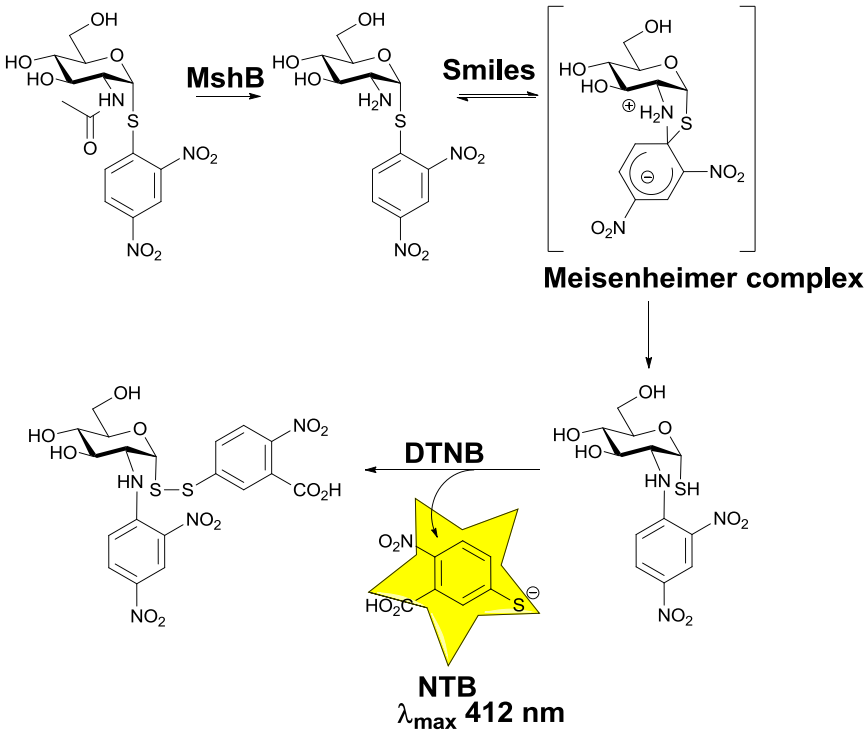

Fig. 4.5: The MshB catalysed reaction as analysed by DTNB assay [9].

The problem with this assay is the low solubility of the alternative substrate, 2,4DNP-α-*S*-GlcNAc, which must be dissolved in DMSO. In addition, it is a poor substrate alternative, requiring high concentrations to show activity. This combination makes it impossible to use to conduct kinetic studies of MshB as the substrate concentration required to saturate the enzyme is too high and would require high levels of DMSO to keep it in solution. In addition, a lag period is observed during the initial stages of the reaction, assumed to be due to intramolecular S→N Smiles rearrangement taking place. Consequently the assay cannot be used to determine the initial rates of activity accurately.

4.3 Motivation for this study

The main aim of the study described in this chapter was to develop a continuous assay for the metallodeacetylases MshB and BshB that would be suited for use in kinetic characterization and inhibitor screening. Specifically, the goal was to establish a method whereby the acetate produced during the course of the reaction could be quantified by through coupling to enzymes that utilise acetate as a substrate. To achieve this, two enzymes that utilise acetate as natural substrate were chosen as possible candidates for the coupling enzyme: acetate kinase and acetyl-CoA synthase. In both cases, the reactions catalysed by these two enzymes can be linked to other enzymes that finally results in the consumption or production of NADH, which be followed spectrophotometrically. The amount of NADH consumed can then correlated to the amount of acetate produced. However, this can be done in several ways.

In this study three different routes were investigated; these were based on previously published assays for other deacetylases [18-20]. The first route entails coupling to the enzyme acetyl-CoA synthetase (ACS), which is then linked to myokinase, pyruvate kinase (PK) and lactate dehydrogenase (LDH) (Figure 4.6) [19]. The second route couples to citrate synthase (CS) and malate dehydrogenase (MDH) (Figure 4.7) [20]. In the last route acetate production is coupled to acetate kinase (AK), and subsequently to phosphotransacetylase (PTA) and then to PK/LDH (Figure 4.8) (PTA is added to prevent the forward reaction of AK, see Figure 4.12). Here we describe the results of our studies to establish the potential and utility of these various systems for application as a robust continuous assay for MshB and BshB activity by using the MshB reaction with GlcNAc as substrate as test case.

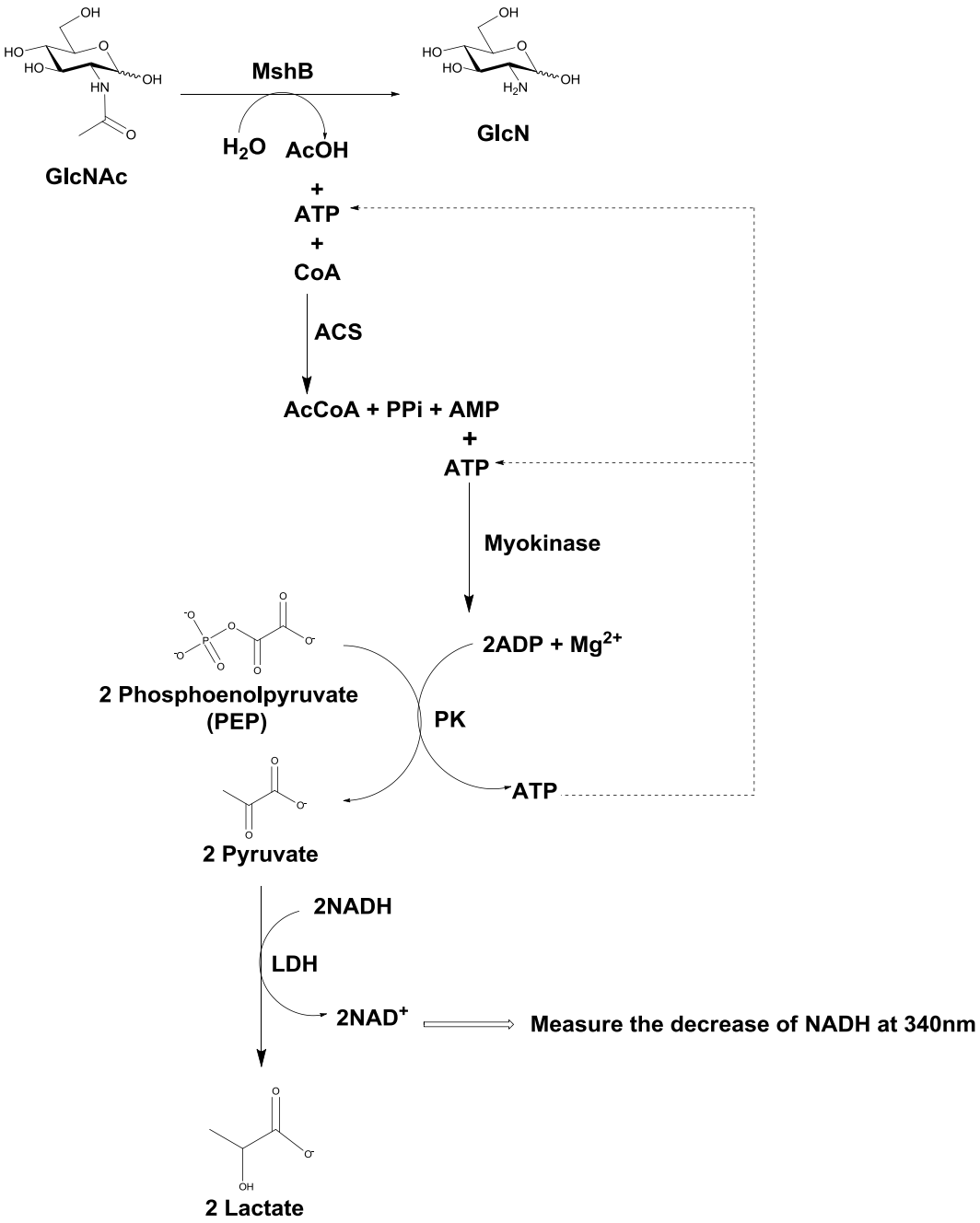

Fig. 4.6: The proposed continuous deacetylase assay based on the coupling of acetate production to acetyl-CoA synthetase and a subsequent cascade ending in the oxidation of NADH through myokinase [19].

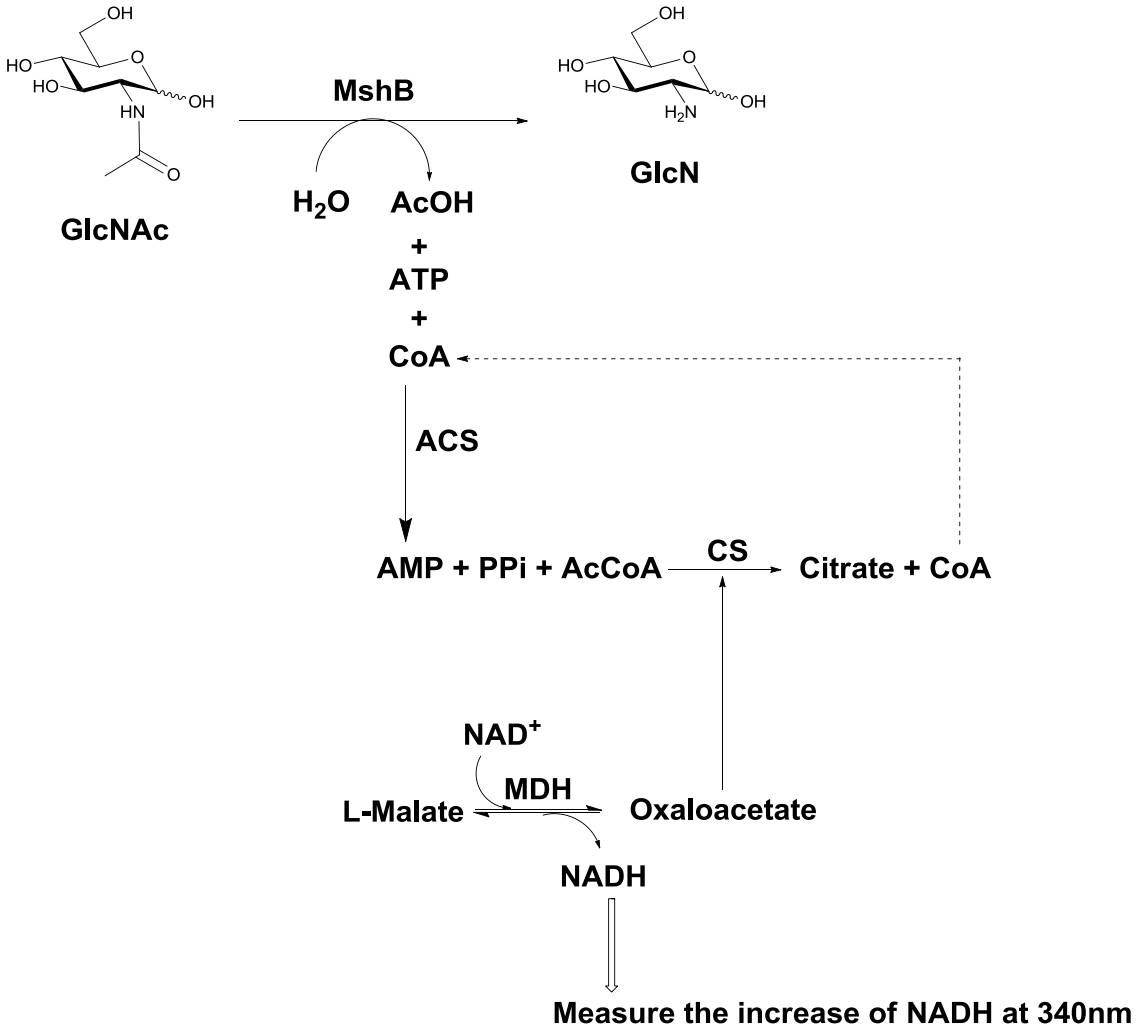

Fig. 4.7 The proposed continuous deacetylase assay based on the coupling of acetate production to acetyl-CoA synthetase and a subsequent cascade ending in the oxidation of NADH through citrate synthetase [20].

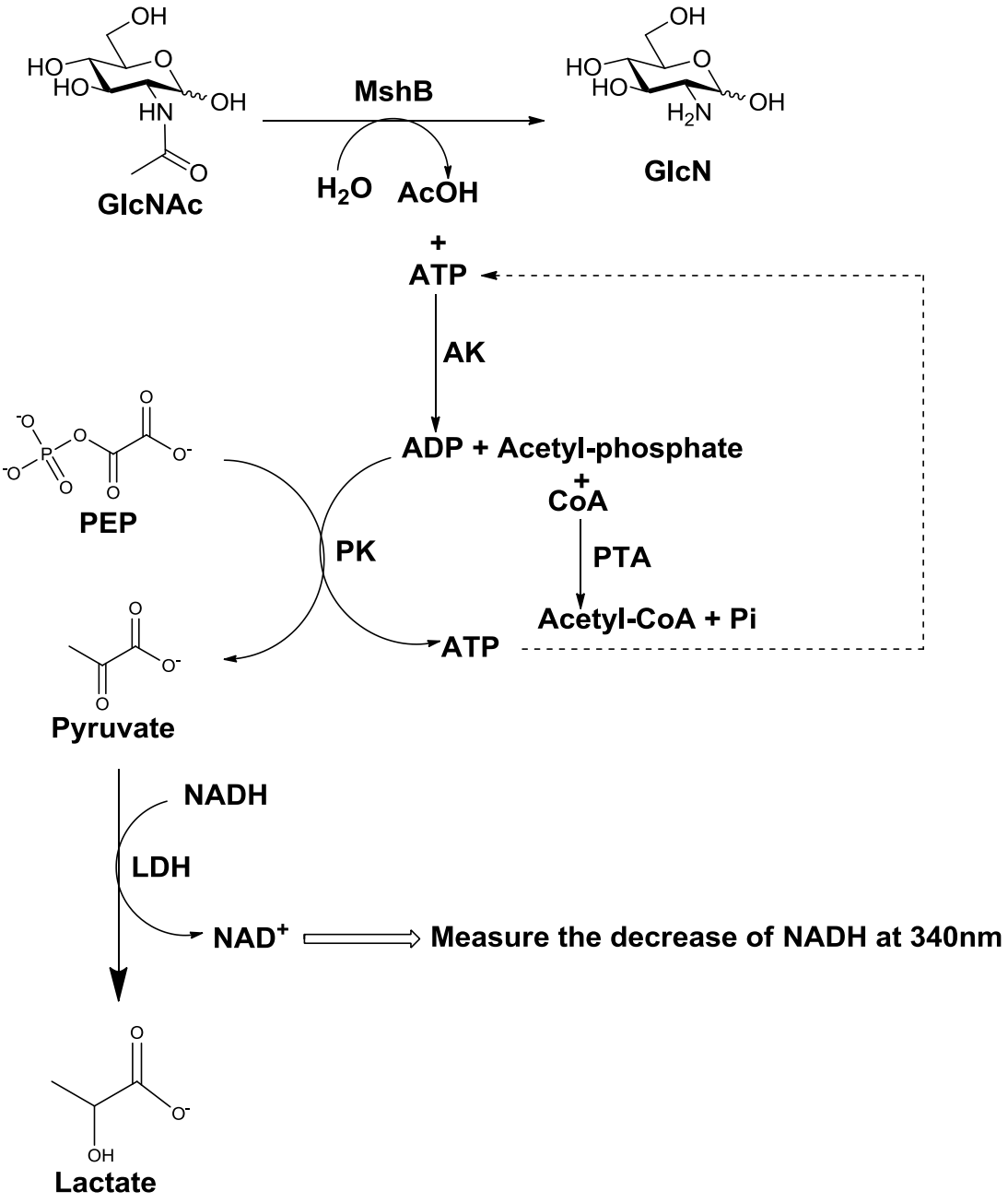

Fig. 4.8: The proposed continuous deacetylase assay based on the coupling of acetate production to acetate kinase and a subsequent cascade ending in the oxidation of NADH. This assay is commercially available in kit form [www.megazyme.com: K-ACETRM].

4.4 Results and discussion

4.4.1 Evaluation of a continuous assay with ACS, PK, LDH and myokinase as coupling enzymes

The main physiological function of ACS is to activate acetate by forming AcCoA [18]. The first ACS that was discovered was the ADP-forming version (EC: 6.2.1.13) from *Entamoeba histolytica*, discovered in 1977 by *Reeves et al.*[21]. Since then, many ACS enzymes have been discovered from different microorganisms, such as *Griardia lamblia*, *Pyroccus furiosus* among others [22, 23]. The AMP–forming version of ACS (EC: 6.2.1.1) belongs to the acyl-adenylate-forming enzyme superfamily [23]. Its reaction occurs in two steps (Figure 4.9) and is assayed by the detection of unreacted CoA or the AMP produced as by-product [19, 24, 25]. The former is detected using DTNB (Ellman's reagent) that is tracked examined spectrophotometrically at 420 nm [19, 24]. Detection by means of AMP-formation utilises the coupling of myokinase to PK and LDH which allows the reaction to be followed by the oxidation of NADH [19]. In the context of deacetylase assays, ACS was utilised by *Wolfson et al*. for the characterisation of histone deacetylase [20]. The same assay was used by *Krämer et al.* in 2016 to characterise the reaction of acetylpolyamine amidohydrolases [26].

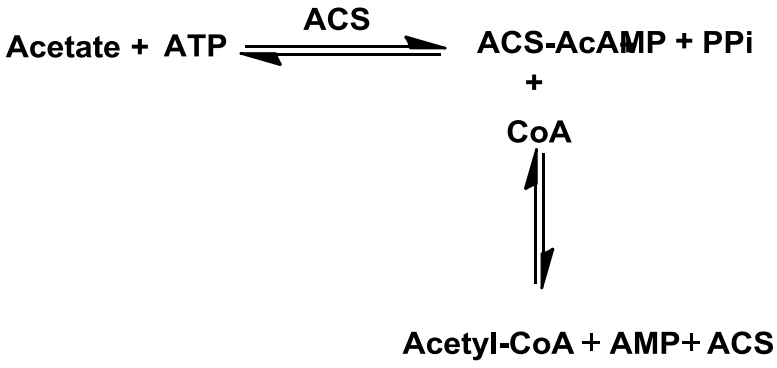

Fig. 4.9: The reaction catalysed by AMP-forming ACS enzymes [25].

In 1987 *Patel et al.* used PK, LDH and myokinase as coupling enzymes in an assay (Figure 4.7) of ACS from yeast in which various carboxylic acid compounds were screened as alternative substrates [19]. Several carboxylic acid compounds such as propanoic acid and 2-propanoic acid amongst other were identified as alternative

substrates for the enzyme. Using this report as starting point, we set out to establish if this assay could be used for determining MshB activity. Six test reactions were set up to ensure that the observed activity could be attributed to MshB, and not to background activity of one of the coupling enzymes (see Table 4.1 for the different reactions and expected result for each). The results of these tests are shown in Figure 4.10. This shows that even in the case of negative controls (i.e. in the absence of coupling enzymes), high levels of background activities were observed. The background activity was observed for all reactions containing ACS and myokinase (Blank, positive control, experiment and negative control 3). The amount of NADH in these reactions was very low in relation to the ones without ACS (negative control 1) or myokinase (negative control 2). This indicates that the coupling reactions had already occurred, depleting the NADH that was present in the master mix at the start of the reactions. For the reaction without ACS (negative control 1) the NADH level remained constant meaning that no reaction was taking place as expected. In the absence of myokinase (negative control 2) the NADH level decreased slowly indicating a slow background activity, most likely due to the presence of AMP in the ATP used in the reaction mix.

Table 4.1: Test reactions for ACS-based MshB assay with expected results for each.

Reaction types	Content of the reaction	Expected results
Blank	Everything except GlcNAc	No change in NADH
Experiment	MshB reactions	Decrease of NADH
Positive control	No MshB, but AcOH added as substrate	Decrease of NADH
Negative control 1	Everything, except ACS	No change in NADH
Negative control 2	Everything, except myokinase	No change in NADH
Negative control 3	Everything, except MshB	No change in NADH

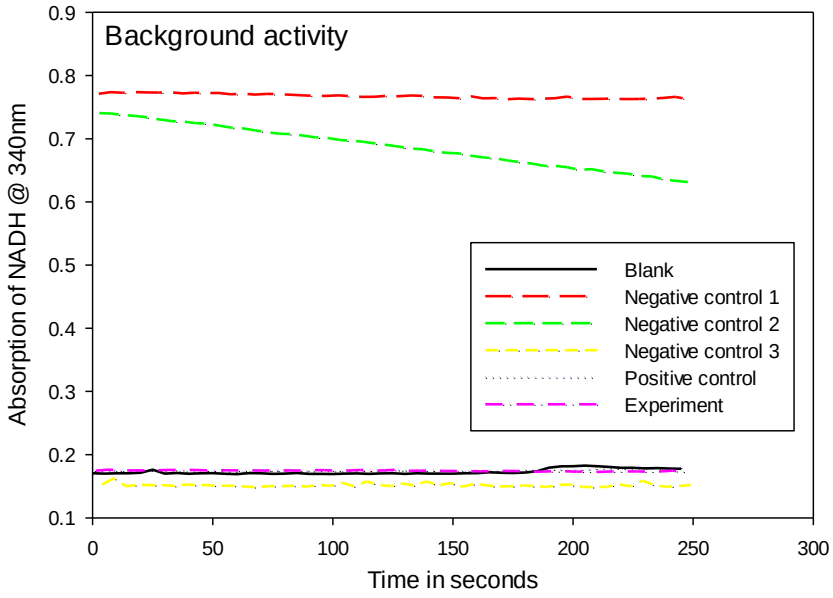

Fig. 4.10: The evaluation of the test reactions set up to evaluate the ACS-based MshB coupled assay (see Table 4.1 for reaction conditions).

To establish the cause of the background activity, the various components of the reaction mixture was sequentially added. The initial test mixture contained buffer with LDH, PK, $MgCl_2$, KCl, NADH and PEP (A). For the next reaction myokinase was added (B), followed ATP (C), and CoA (D). ACS was added last (E). The addition of C, ATP, results is a slight decrease which stabilised over time. This indicated that the ATP is contaminated with some trace of ADP, that is, some of ATP has been hydrolysed into ADP and Pi. The addition of ACS resulted (E) in an immediate decrease of NADH, even though no acetate was present the mixture (Figure 4.11). This clearly indicates that the ACS used in this assay is responsible for the background activity. This is most likely due to the commercial ACS preparation is not pure, that is, the ACS mixture might contain other enzymes in the mixture that are either promoting the PK reaction, LDH reaction or they can reduce NADH to NAD^+. Since the further development of this assay required a high purity ACS preparation that was not accessible at this time, our attention moved to other options for continuous deacetylase assays.

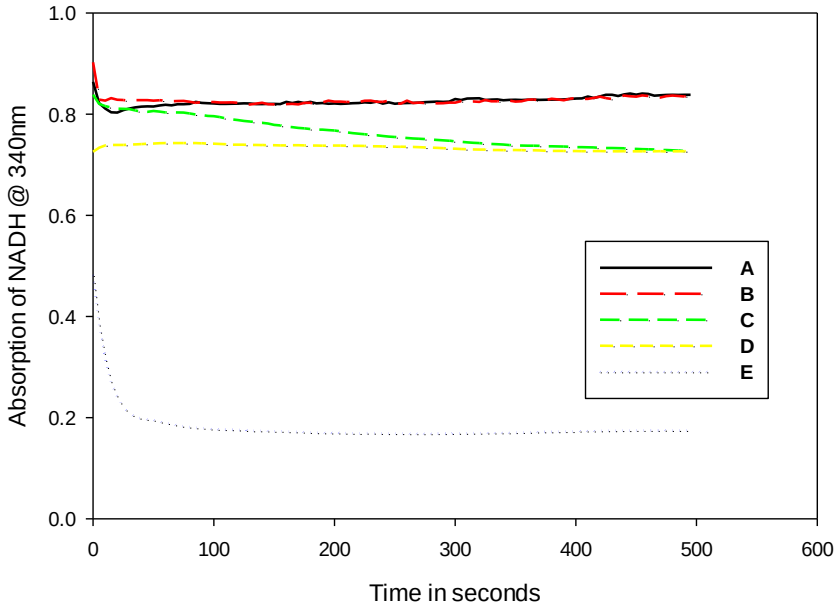

Fig. 4.11: Identification of the background activity through sequential addition of the reaction reagents. A: buffer with LDH, PK, $MgCl_2$, KCl, NADH and PEP; B: myokinase added to A; C: ATP added to B; D: CoA added to C; E: ACS added to D.

4.4.2. Evaluation of a continuous assay with ACS, CS and MDH as coupling enzymes

Wolfson *et al.* and Krämer *et al.* used an assay in which ACS is coupled to CS and MDH to characterise the reaction of histone deacetylase and acetylpolyamine amidohydrolases, respectively [20, 26]. In both cases they were able to detect the amount of the acetate released in a continuous manner. However, when the same assay was applied to determine the activity of MshB using the conditions reported in these studies, no activity could be observed even after several reactions, not even background activity. Subsequently several acetate compounds (such sodium acetate) were used in what would be positive control reactions. However, no activity was detected. Modifying the ratio of coupling enzymes within the assay also did not result in any activity. It was assumed that the no activity results could be associated with the linking enzymes (CS and MDH), that is, maybe they are inactive. Studies on evaluating the activity of CS and MDH were never done because if the problem could be resolved there was still the problem of the background activity of commercial ACS. Wolfson *et al.* indicated that a highly purified recombinants ACS is required for the use of such assay in order to decrease the background activity [20].

The accessible of a highly purified recombinants ACS at this time was not feasible. Consequently the further investigation of this route was also terminated.

4.4.3 Evaluation of a continuous assay with AK, PTA, PK, and LDH as coupling enzymes

Acetate kinase is a reversible phosphoryl transfer enzyme that transfers and activated phosphoryl group to acetate to generate acetyl-phosphate and either ADP or Pi as a by-product [27]. The phosphoryl donor can either be diphosphate or ATP, with the reactions being catalysed by acetate kinase (EC: 2.7.2.12) and acetate kinase (EC: 2.7.2.1), respectively (Figure 4.12). Acetate kinase was discovered by Lipmann in 1944 [28] and is a member of the ASKHA (Acetate and Sugar Kinases; Hsp70, Actin) phosphotransferase superfamily [27, 29]. Its reaction is characterised either through the use of enzyme-coupled assays [30] or through the hydroxamate assay, with the latter being derived from the Lipmann-Tuttle and Rose methods [31, 32]. The hydroxamate assay involves the use of hydroxylamine that reacts with acetyl-phosphate to form acetyl hydroxamate; this produces a coloured complex with ferric ions [27, 31-33]. The enzyme-coupled assay uses the PK and LDH enzymes to detect the ADP produced during the reaction. Currently, acetate kinase is utilised in the acetic acid kit from Megazyme to determine the amount of acetic acid in food.

Fig. 4.12: The reversible reaction of AK [27].

Initial screening of the acetate kinase-based assay as a potential continuous assay for MshB deacetylase activity again involved inspection to determine any background activity or interference within the assay. GlcNAc was selected as the substrate, and was used in the assay at different concentration (0 - 250 mM) in the absence of MshB. All reactions except the one with no GlcNAc (0 mM) gave background activity (Figure 4.13). Moreover, the rate of NADH reduction was found to correlate with the GlcNAc concentration, that is, the higher the amount of GlcNAc used the faster the rate of NADH reduction was found to be. This suggested that the GlcNAc preparation contained acetic acid as contaminant. The GlcNAc preparation

was therefore subjected to several rounds of freeze drying in an attempt to remove the acetic acid, after which the test was repeated.

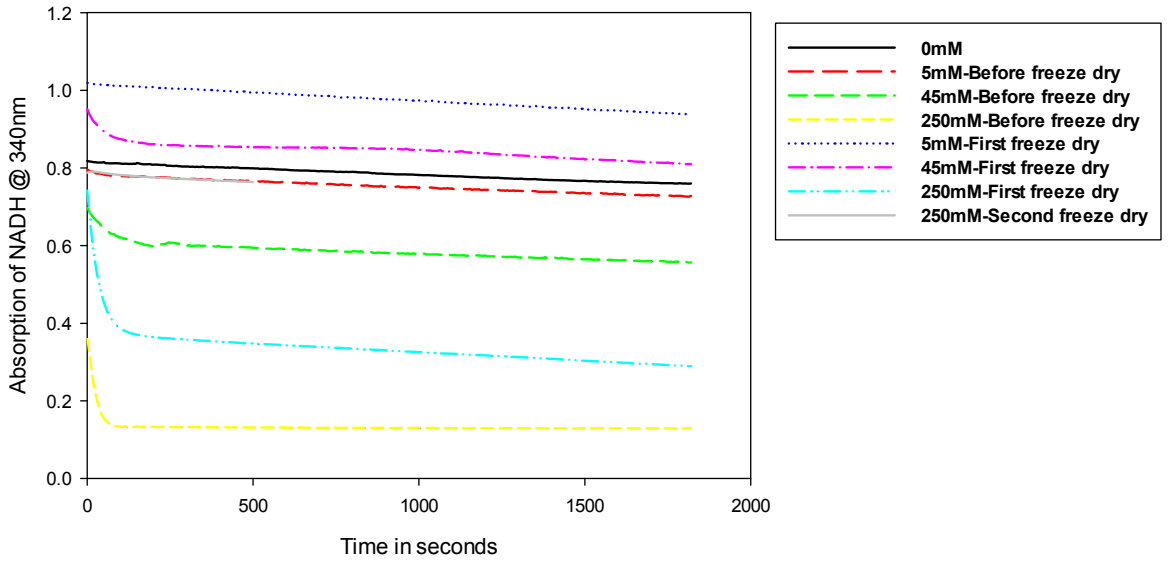

Fig. 4.13: Background activity of acetate kinase with GlcNAc as a substrate (in the absence of MshB).

The rate of reduction of NADH was reduced dramatically in reactions in which freeze dried GlcNAc was used. GlcNAc was therefore subjected to a further round of freeze drying; the GlcNAc that was purified in this manner gave no background when tested at 250 mM (Figure 4.13). The successful removal of acetic acid from GlcNAc made it possible to investigate the acetate kinase-based assay for use as an MshB assay.

The acetate kinase-based assay was evaluated by using it to determine the MshB kinetic parameters with GlcNAc as substrate, and to compare the results to published values. The kinetic study was done with the pure GlcNAc in a concentration range from 0–250 mM. The acetate kinase-based assay gave reasonable MshB activity profiles (Figure 4.14A), which were hyperbolic in nature, not showing the sigmoidal profiles previously observed for compounds **12** and **13** when the FSA assay was used (see chapter 3). The kinetic parameters obtained by fitting the Michaelis-Menten equation to the data differed from the ones published by Huang *et al*., which was obtained using the FSA assay (Table 4.2) [2]. The K_M obtained from the acetate kinase assay was five-fold higher compared to the

published value, while the k_{cat} value was lower, although by a factor less than two. However, it should be pointed out that the data in the study by Huang *et al*. was obtained using the *M. smegmatis* MshB, while our studies were performed with the *M. tuberculosis* enzyme.

To exclude the possibility that the low activity could due to the coupling enzymes, i.e. that one of these enzyme are rate limiting, the assay was performed with different concentrations of MshB (0.5 µM, 1.0 µM, 2.0 µM and 2.5 µM) at one concentration of GlcNAc (38 mM). The activity of MshB was found to increase linearly with the amount of enzyme used indicating that the coupling enzymes are not a limiting factor in the assay (Figure 4.15). A linear equation (**y = mx + c**) was fitted through the data to producing the following equation **y = 0.8561x + 0.0126 (R^2 = 0.9865)**.

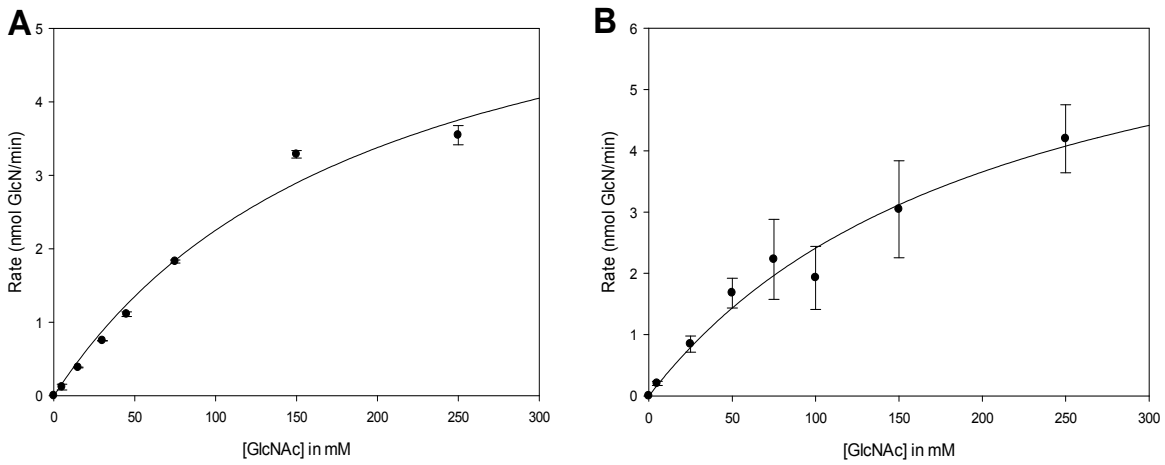

Fig. 4.14: Activity profiles for MshB with GlcNAc: **Panel A** is a continuous assay done through the AK-PTA-PK-LDH coupling-enzyme assay with a fit of R^2 = 0.9800. **Panel B** is a discontinuous assay done through FSA derivatization-based assay with a fit of R^2 0.9006 (The standard curve, Figure 3.10 in Chapter 3, was used to convert the fluorescence units into [GlcN]). The data represent average rate of MshB at various concentration of GlcNAc with the error bars representing standard deviation. Activity assays were performed using a range of concentrations (0 mM - 250 mM) of the different compounds and were in triplicate for FSA assay and duplicate for AK-PTA-PK-LDH assay coupling-enzyme. The non-linear Michaelis-Menten equation **($v=V_{max}*S/(K_M+S)$** was fitted through the data.

Table 4.2: Kinetic study of MshB with GlcNAc as substrate through different assays

Kinetic parameters	FSA assay (published)*	AK-PTA-PK-LDH coupling assay	FSA assay
K_M (mM)	38 ± 4.0	199 ± 38	213 ± 74
k_{cat} (min^{-1})	46 ± 2.2	25 ± 2.7	30 ± 6.2.
k_{cat}/K_M ($mM^{-1}.min^{-1}$)	1.2 ± 0.08	0.125 ± 0.027	0.141 ± 0.057

* Obtained from Huang *et al* [2] ; these data are for the *M. smegmatis* MshB enzyme.

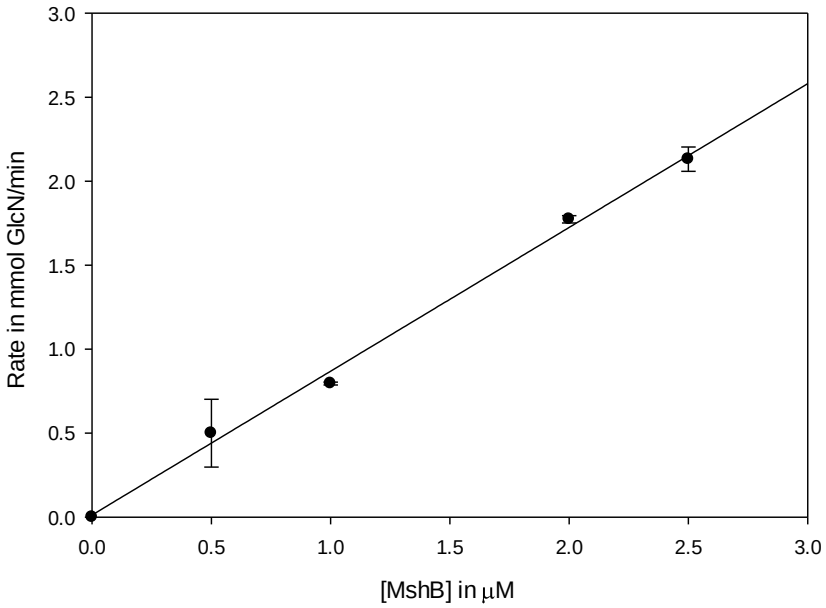

Fig. 4.15: Activity profile of MshB at various concentration with GlcNAc constant at 38mM. The activity was done through the AK-PTA-PK-LDH assay coupling-enzyme. The data represent average activity rate of MshB at various concentration with the error bars representing standard deviation. Activity assays were performed using a range of concentrations (0–2.5 mM) of the MshB and were done in triplicate. A linear equation (**y = mx + x**) was fitted through the data.

As the coupling enzymes were excluded as a possible source of error, we considered that the difference between the kinetic parameters observed during this study and those reported by Huang *et al.* could be due to the FSA assay used in the previous studies. We therefore also obtained an MshB activity profile using the FSA assay as described by Huang *et al.* (Figure 4.14B). The kinetic parameters obtained in this manner agreed very well with those from the acetate kinase-based assay (Table 4.2). This suggested that the difference in the kinetic parameters reported for MshB by *Huang et al.* and those obtained here is likely due to the difference in source of the enzymes, as the published data was from the *Mycobacterium smegmatis* enzyme [2], while the *M. tuberculosis* MshB was used in this study. Taken together, the results seemed to indicate that the acetate kinase-based assay could be used to characterise MshB activity with any substrate as long as there is no trace of acetic acid is present. To evaluate such claim α-GlcNAc-OMe was used as substrate in reactions with MshB, and the deacetylase activity tracked using both the acetate kinase-based and FSA assays for direct comparison.

Activity profiles for MshB with α-GlcNAc-OMe using the acetate kinase-based and FSA assays were obtained without any problems (Figure 4.16). The fit of the non-liner Michaelis-Menten equation through the FSA assay data was not a good fit (R^2 = 0.8795) in relation to the AK-PTA-PK-LDH coupling assay (R^2 = 0.9813), hence the large difference between the kinetic parameters (Table 4.3). The kinetic parameters obtained from FSA assay active profiles are very inconclusive due to the large error margin. If one has to take the error into account FSA has a high *kcat* and low K_M in relation to acetate kinase-based assay; however such account cannot be done because the error margin is so large. The data scatted of the FSA assay could be assumed to be due to the inaccessibility of the free amine group of the reaction product by the FSA, that is, the α-anomeric configuration of α-GlcN-OMe might be producing steric hindrance at the anomeric centre making it difficult for the FSA to reach the amine group (amine group and methyl group are facing the same direction) or because the standard curve used to convert the fluorescence units into α-GlcN-OMe concentration was generated from glucosamine and not α-GlcN-OMe. To prove such assumption a standard curve generated from α-GlcN-OMe would be required of which it is very challenging due to the formation of the α-configuration of the glycoside that impedes access the free amine group on the 2-position.

Looking at the activity profiles (Figure 4.14 and 4.16) of MshB with the different substrates (α-GlcNAc-OMeand α-GlcNAc) it is clear from both profiles that AK-PTA-PK-LDH coupling assay seem to the best assay to characterise MshB reaction compared to FSA assay. Unlike FSA assay, the AK-PTA-PK-LDH coupling assay relies on the acetate produced during the reaction without the need of generating a standard curve or concerns about the possibility of steric hindrance interfering in the association between the derivatization agent and the reaction product. As such the AK-PTA-PK-LDH coupling assay can be applied to wider range of substrate.

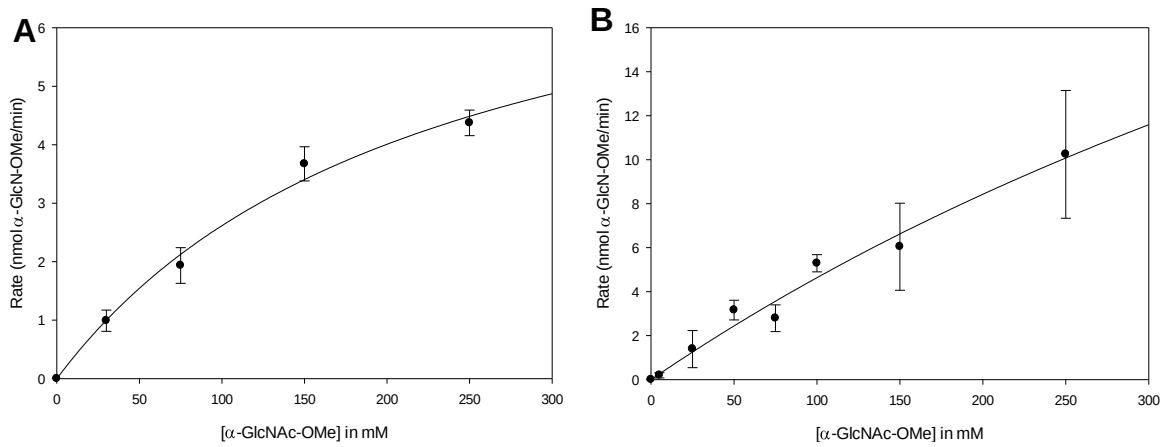

Fig. 4.16: Activity profiles for MshB with α-GlcNAc-OMe: **Panel A** is a continuous assay done through the AK-PTA-PK-LDH coupling-enzyme assay with a fit of R^2 0.9813. **Panel B** is a discontinuous assay done through FSA derivatization-based assay with a fit of R^2 0.8795 (The standard curve, Figure 3.10 in Chapter 3, was used to convert the fluorescence units into [α-GlcN-OMe]). The data represent the average rate of MshB at various concentration of α-GlcNAc-OMe with the error bars representing standard deviation. Activity assays were performed using a range of concentrations (0–250 mM) of the different compounds and were in triplicate for FSA assay and duplicate for AK-PTA-PK-LDH assay coupling-enzyme. The non-liner Michaelis-Menten equation **($v=V_{max}*S/(K_M+S)$)** was fitted through the data.

Table 4.3. Kinetic study of MshB with α-GlcNAc-OMe through different assays

Kinetic parameters	AK-PTA-PK-LDH coupling assay	FSA assay
K_M (mM)	227 ± 61	903 ± 837
k_{cat} (min^{-1})	32 ± 4.9	186 ± 143
k_{cat}/K_M ($mM^{-1}.min^{-1}$)	0.14 ± 0.043	0.20 ± 0.24

4.5 Summary and conclusion

Three different continuous deacetylase assays that is, ACS-PK-LDH-myokinase, ACS-CS-MDH and AK-PTA-PK-LDH were tested as potential candidates for characterisation of MshB activity. Of the three, ACS-PK-LDH-myokinase assay showed background activity as a result of the main coupling enzyme (ACS), while the ACS-CS-MDH assay show no activity at all. The AK-PTA-PK-LDH assay showed that MshB reaction can be followed through the detection of the acetate produced during the reaction, as long as there is no trace of acetate/acetic acid in the substrate being used. AK-PTA-PK-LDH assay was also found to be a better assay in relation to the FSA assay, that is, looking at the activity profiles for MshB with GlcNAc and α-GlcNAc-OMe through the two assays (Figure 4.15 and Figure 4.16) it clearly shows that acetate kinase-based assay is a better assay compared to the FSA assay. The data points of the acetate kinase-based assay had small error margin to those of FSA assay and the non-liner Michaelis-Menten equation was a better fit through the data. The initial rate can be determined without any problem, since AK-PTA-PK-LDH assay is a continuous assay which depends on the acetate formation and not the amine group, which means no standard of the reaction, is required for the generation of the standard curve.

Looking at the kinetic parameters of AK-PTA-PK-LDH assay and FSA assay especially that of the α-GlcNAc-OMe it is clear that FSA assay is not the best assay to characterise reactions wherein α-GlcNAc-based glycosides are used as alternative substrate. This might explain the undetectable activity of MshB with α-GlcNAc-N_3 and α-GlcNAc-CyCOH (Figure 3.13 in Chapter 3) at low concentrations and the apparent cooperative kinetic behaviour that was observed, that is the observed cooperativity was due to the assay used and not due to substrate. Due to low amount of α-GlcNAc-N_3 and α-GlcNAc-CyCOH the activity of MshB with these compounds could not be tested. Lastly AK-PTA-PK-LDH assay is a better continuous assay than the DTNB-based assay because does not require the substrate to dissolve in DMSO or the formation of any secondary product (Smiles-rearrangement). In conclusion a continuous assay for metallo-deacetylase enzyme was found which allowed the characterisation of the reaction as it occurs through the determination of the acetate produced during the reaction.

4.6 Material and methods

All the chemicals used were purchased from Sigma-Aldrich unless stated otherwise except, GlcNAc which was brought from Carbosynth and α-GlcNAc-OMe chemical synthesised using the previously described method by *Gao et al.* [34] and *Neuberger et al.* [35]. The assays were done using Greiner Bio-One polystyrene flat-bottomed 96-well plate, that is clear plate for the different routes assay (ACS-PK-LDH-myokinase; ACS-CS-MDH and AK-PTA-PK-LDH) and black plates were for FSA assay. Varioskan multimode reader (Thermo Scientific) was used to measure the fluorometric and spectrophotometric readings. All enzymes were purchased from Sigma-Aldrich, with exception to the ones used within the acetic kit from Megazyme and MshB. MshB was expressed and purified according to Newton et al for [4]. The cultures were grown until the optical density at 600nm (OD_{600}) reached 0.600 and induced with 0.10mM of isopropyl-1-thio-β-D-galactropyranoside (IPTG) for MshB followed by incubation of the cultures overnight at 22°C. This was followed by cell harvest by centrifugation at 17600×*g* for 20 minutes at 10°C. The pellets cells were stored at -20°C until use. The enzymes was purified with automated purification program on ÄKTA*prime* system (Amersham Bioscience) using the previously described methods. SDS-PAGE was used to check the purity of the protein, while Bradford was used to determine the protein concentration.

4.6.1. Studies toward the development of a potential high throughput continuous assay

4.6.1.1. Evaluation of a continuous assay with ACS, PK, LDH and myokinase as coupling enzymes

The use of ACS, PK, LDH and myokinase as coupling enzymes was inspired and conducted in the same way as *Patel et al.* with some modification to it [19]. The reaction mixture (master mix) containing reaction buffer (50mM HEPES, 50mM NaCl, pH 7.5) together with or without coupling enzymes or MshB, see Table 4.1, were incubated at 30°C for 2 minutes. The reaction was initiated by adding reaction mixture into the substrate or water in the case of blank already in a 96 well microplate. The decreasing absorbance of NADH was monitored at 340nm for several minutes.

4.6.1.2 Evaluation of a continuous assay with ACS, CS and MDH as coupling enzymes

The successful application of ACS by *Wolfson et al.* to assay the histone deacetylase reaction with ACS, CS and MDH as coupling enzymes motivated us to utilised ACS, CS and MDH as coupling enzymes for the metallodeacetylase reaction [20]. The same produce used by *Wolfson et al.* was used to characterise MshB reaction with some modification to it for the development of a continuous assay [20]. The reaction mixture (master mix) containing reaction buffer (50mM HEPES, 50mM NaCl, pH 7.5) together with coupling enzymes and MshB was incubated at room temperature for 30 minutes for the equilibration. The reaction was initiated by adding reaction mixture into the substrate or water in the case of blank already in a 96 well microplate. The increasing absorbance of NADH was monitored at 340nm for several minutes at 30°C.

4.6.1.3 Evaluation of a continuous assay with AK, PTA, PK, and LDH as coupling enzymes

The AK, PTA, PK, and LDH as coupling enzymes were done using the acetic kit from Megazme and conducted as per manual state, with some modification to it [megazyme]. All reagents were added together including MshB and they were incubated at 30°C for 2 minutes before adding reaction mixture into the substrate or water in the case of blank already in a 96 well microplate. The decreasing absorbance of NADH was monitored at 340nm for several minutes at 30°C.

4.6.2 FSA assay

FSA was done in the same as in Chapter 3, see section 3.3.2 of Chapter 3.

4.7 References

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Chapter 5:
Conclusion and Future work

5.1 Summary of results achieved

In this study various α-thioglycoligases that were derived from the *Cp*GH89 α-*N*-acetylglucosaminidase were characterised for their suitability as biocatalysts in the preparation of α-GlcNAc-based glycosides. Several α-GlcNAc-based glycosides were biosynthesised using these α-thioglycoligases and they were tested as small molecule chaperones (to determine their potential in the treatment of Sanfilippo syndrome) and as alternative substrates or inhibitors of the metallodeacetylase MshB. Finally, a new continuous assay for the metallodeacetylases MshB and BshB was developed. The specific objectives that were outlined in Chapter 1 to achieve this were accomplished as follows:

5.1.1 Objective 1: Characterisation of α-thioglycoligases derived from the α-*N*-acetylglucosaminidase *Cp*GH89 for the biocatalytic preparation of α-GlcNAc-based glycosides

Although several glycosidase enzymes have been converted into thioglycoligases, only a few of them promote the formation of the α-thioglycosidic bond [1]. In addition, none of them promote the formation of α-*N*-acetyl-thioglycosides. We generated α-*N*-acetyl-thioglycosides using α-thioglycoligases derived from an α-*N*-acetyl-glucosaminidase. The chemical rescue of the α-thioglycoligases with sodium azide not only showed that the activity of these mutant glycosidases can be restored by the addition of an external nucleophile, but also for the first time allowed for the synthesis of α-GlcNAc-N_3. The enzymes' α-thioglycoligase activity was evaluated with various donors (2,4DNP-α-GlcNAc, 4NP-α-GlcNAc and 2NP-α-GlcNAc) and several thiol molecules as acceptors. No thioglycoligation reactions were observed with 4NP-α-GlcNAc as donor with any of the α-thioglycoligases tested, while 2,4DNP-α-GlcNAc promoted thioglycoligation reactions with some of them. However, 2,4DNP-α-GlcNAc was also found to be more susceptible to hydrolysis due to the stability of its leaving group. We found that the thioglycoligation reaction was best promoted with 2NP-α-GlcNAc as glycosyl donor and with thiol molecules containing

a thiol group having p*Ka* values ranging between 3 and 8. In addition, specific α-thioglycoligases, especially *Cp*GH89-E438A and *Cp*GH89-E438S, also promoted the thioglycoligation reaction on a preparative scale, which allowed the purification and characterisation of the enzymatic reaction products (Table 2.2). The generated α-GlcNAc-based thioglycosides and α-GlcNAc-N_3 were tested for their inhibition and stabilization of *Cp*GH89. Of the tested glycosides, GlcNAc-*S*-CH_2CO_2Me and GlcNAc-*S*-CH_2CO_2Et showed the highest inhibition with 79% and 62% of residual activity respectively. No inhibition was observed with α-GlcNAc-N_3. However, during the stabilization study α-GlcNAc-N_3 showed the best stabilizing effect. This demonstrated that this compound could be a lead in the development of potential small molecule chaperones for the treatment of Sanfilippo syndrome since it does not inhibit the activity of the wild type enzyme, but increases its stability.

5.1.2 Objective 2: Chemo-enzymatic synthesis and characterization of GlcNAc-based glycosides as alternative substrates or inhibitors of MshB and/or BshB

Several GlcNAc-based thioglycosides that could potentially act as alternative substrates of MshB and/or BshB were synthesized chemo-enzymatically (Table 3.2). Chemical modification of the biosynthesised α-GlcNAc-based thioglycosides α-GlcNAc-SMal$(OMe)_2$ and α-GlcNAc-*S*-CH_2CO_2Me allowed for the synthesis of the BshB substrate analogues α-GlcNAc-*S*Mal and α-GlcNAc-*S*CH_2CO_2H. In addition, α-GlcNAc triazoles were generated from biocatalytically prepared α-GlcNAc-N_3 through “Click” chemistry. These α-GlcNAc triazoles and α-GlcNAc-N_3 were found to show higher MshB deacetylation activity than the best alternative substrates currently in use (Figure 3.11). The preliminary kinetic study also indicated that an α-GlcNAc triazole (compound **12)** and α-GlcNAc-N_3 (**13**) are better alternative substrates than GlcNAc. Unfortunately, the BshB deacetylation activity against various compounds couldn’t be tested since active BshB could not be prepared in this study.

5.1.3 Objective 3: Development of a continuous assay suitable for the characterization of the metallo-deacetylases, MshB and BshB

With the exception of one assay described by Lamprecht *et al*. [2], all the assays that are currently available and used to characterise BshB and MshB activity are discontinuous assays [3-7]. However, such assays are less than ideal since all of them make use of derivatizing reagents which adds complexity, while some utilise compounds that are classified as toxic. Moreover, as they are all endpoint assays the progress of the reactions cannot be followed without performing an assay for each time point. On the other hand, the continuous assay reported by Lamprecht *et al.* utilizes as substrate a compound that shows relatively low activity, and it can only be dissolved DMSO, thus, the concentration range of substrate that can be used is limited.

In our attempts to develop a continuous assay suitable for use in high-throughput screens we evaluated several potential coupled enzyme systems, including the ACS-PK-LDH-myokinase [8], ACS-CS-MDH [9] and AK-PTA-PK-LDH [www.megazyme.com: K-ACETRM] systems for their potential and suitability as continuous assays for MshB's deacetylation activity with GlcNAc as substrate. Of the three different systems investigated only the AK-PTA-PK-LDH system gave reproducible results. The background activity observed with the ACS-PK-LDH-myokinase system was too high and no activity was observed with ACS-CS-MDH system, leading to these systems not be considered any further. Using the newly developed assay, we were able to characterise MshB activity with GlcNAc and α-GlcNAc-OMe as substrates. In addition, the activity profile of MshB with different substrates was better when using the AK-PTA-PK-LDH coupled assay compared to the discontinuous end-point assay that relies on fluorescamine to react with the free amine of such as compound. The AK-PTA-PK-LDH system is thus a good candidate for utilization as an assay for the characterization of this particular metallo-deacetylase.

5.2 Conclusion

Overall the main goal of this project was attained through the vigilant evaluation of the available thioglycoligase enzymes under several reaction conditions. The α-GlcNAc-based glycosides generated in this manner have some potential for development of small molecular chaperones for the treatment of Sanfilippo syndrome. In addition, the AK-PTA-PK-LDH continuous assay system was shown to be a viable method for characterizing MshB activity.

This project opened the door in the search for other α-*N*-acetylglucosaminidases that can also be converted into α-thioglycoligases with other acceptor specificities, and which can therefore be used for the generation a different variety of α-GlcNAc-based thioglycosides. Furthermore, we developed a way to synthesize α-GlcNAc-N_3 easily using the α-thioglycoligase *Cp*GH89 as biocatalyst, making it available so that its use in other applications can be explored further.

5.3 Future work

5.3.1 Engineering and development of and α-thioglycoligase and conditions suitable for thioglycoligation using a larger scope of acceptors

The α-thioglycoligases developed in this study only promote the thioglycoligation reaction with thio-molecules that had a pK_a value below 8. Thio-molecules with a thiol groups having pK_a values higher than 8 were unable to act as thio-acceptors since a thiolate anion could not be generated under the reaction conditions used. Future work involves finding reaction conditions that may allow thioglycoligation with thiol-molecules that have thiol group with higher pK_a values, and without hydrolysis of the glycosyl donor.

5.3.2 Preliminary characterisation of MshB with the newly chemo-enzymatic synthesised α-GlcNAc-based glycosides

The several α-GlcNAc-based glycosides prepared for the first time in this study using the reported biocatalytic method were tested as alternative substrates of MshB using the FSA assay. Of the tested compounds, compounds **3**, **8**, **12** and **13** were found to have higher deacetylation activity with MshB than GlcNAc, the alternative substrate

mostly used in current studies (Figure 3.11). In addition, a preliminary kinetic study of MshB using compounds **12** and **13** suggested that they are better alternative substrates for the enzyme than GlcNAc. Future studies will involve generating more of the α-GlcNAc-based thioglycosides and azides/triazoles that showed high MshB deacetylation activity by using the methods that were developed as described in Chapter 2. These compounds will be characterised with the newly validated AK-PTA-PK-LDH-based assay of deacetylases, such MshB. This will allow the full kinetic characterisation of the activity of MshB and/or BshB towards such compounds; this will aid in the identification of alternate MshB substrates other than GlcNAc. Lastly, further studies will be conducted to isolate the BshB enzyme in its active form so that it too can be characterised with the different α-GlcNAc-based glycosides prepared in this study to date.

5.3.3 The validation of the newly developed continuous deacetylase assay for use in inhibitor screening and characterization

The availability of a new continuous assay for the deactylases MshB and BshB allows for more accurate determination of the initial rates of the reactions catalyzed by these enzymes. It also greatly facilitates any inhibitor screening and characterization processes. Future work will involve using the AK-PTA-PK-LDH assay identified as the best alternative for characterisation of MshB activity with the various GlcNAc-based glycosides, azides and triazoles produced in others studies (including those described in this thesis). It will also be used to establish if the system is suitable for inhibitor screening, as this would facilitate efforts to identify a new MshB and/or BshB inhibitor from existing compound libraries.

5.4 References

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Appendix

Supplementary information supplied in support of publication reproduced in Chapter 2.

SUPPORTING INFORMATION

The α-thioglycoligase derived from a GH89 α-*N*-acetylglucosaminidase synthesizes α-*N*-acetylglucosamine-based glycosides of biomedical interest

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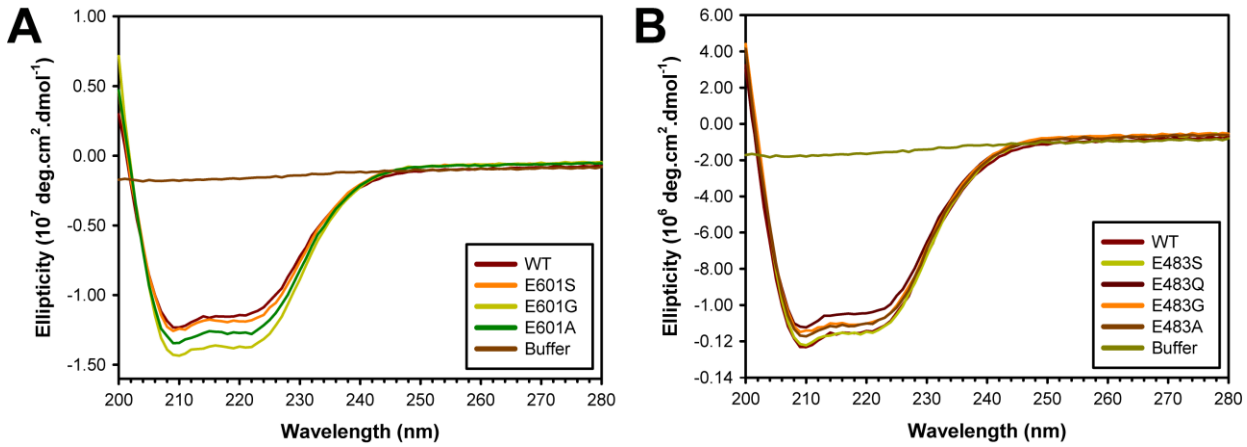


Figure S1. *CpGH89 structure-based stability.* **A**: CD absorption spectra of the buffer, wild type enzyme and its E601 mutants at 25°C showing that all the proteins have similar shapes. **B**: As for A, but for the E483 mutants.

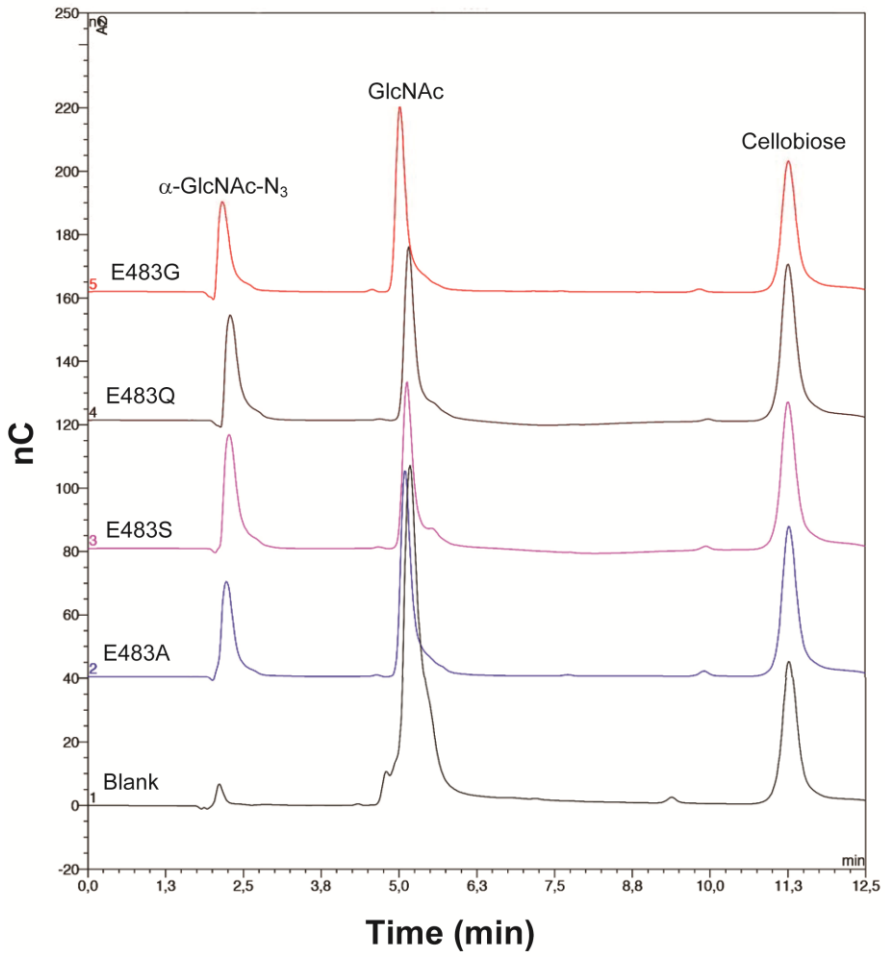


Figure S2. *Chemical rescue of CpGH89–E483A/S/Q/G mutants in the presence of sodium azide.* HPAEC-PAD chromatogram of the reactions performed by the mutants (1.33 μM). The mixtures contained 100 mM sodium phosphate buffer at pH 7.3, 5 mM 2,4DNP-α-GlcNAc, and 250 mM sodium azide. Blank mixtures, containing all the reagents but the enzymes were incubated at the same conditions and used to test the spontaneous hydrolysis of the donor. Reactions were incubated for 16h at 37°C. Cellobiose was used as internal standard.

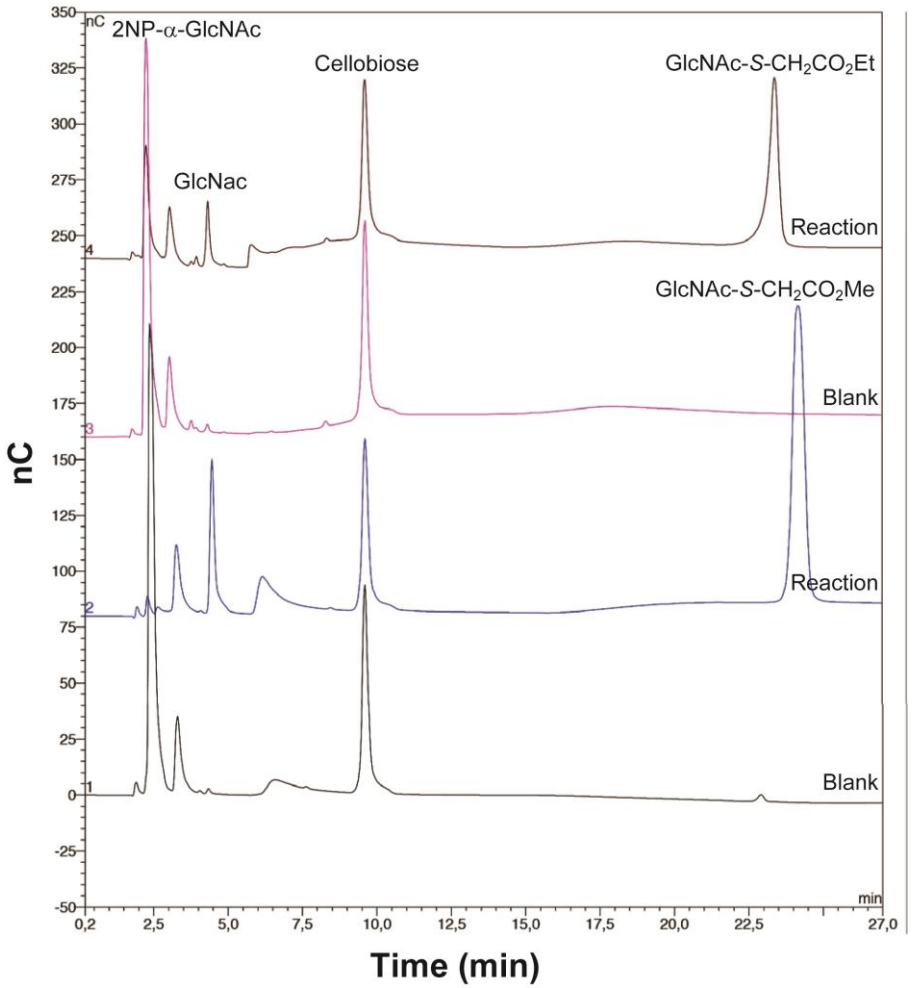


Figure S3. *Thioglycoligase activity of CpGH89-derived acid/base mutants.* HPAEC-PAD chromatograms of the reactions performed by the CpGH89–E483A mutant are shown as case examples. The mixtures contained 100 mM sodium phosphate buffer at pH 8.0, 5 mM 2NP-α-GlcNAc, and 106 mM of either methyl thioglycolate (Me) or ethyl thioglycolate (Et) acceptors. Blank mixtures, containing all the reagents but the enzyme were incubated at the same conditions and used to test the spontaneous hydrolysis of the donor. Reactions were incubated for 48h at 37°C. No side-products could be observed but only unreacted donor. Cellobiose was used as internal standard.

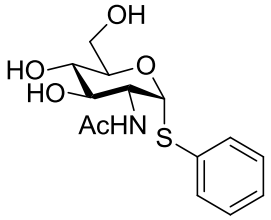
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

1. *NMR and MS analysis methods*

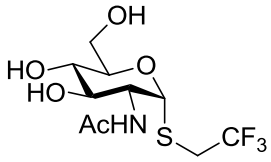
NMR analyses: All ^{1}H and ^{13}C NMR spectra were obtained using a 300 MHz Varian VNMRS (75 MHz for ^{13}C), 400 MHz Varian Unity Inova (100 MHz for ^{13}C) or 600 MHz Varian Unity Inova (150 MHz for ^{13}C) instruments (Varian Inc., Palo Alto, CA, USA) at the Central Analytical Facility (CAF) of Stellenbosch University. All chemical shifts (δ) were referenced to the reported values of Gottlieb (Gottlieb, et al., 1997) using the signal from the residual protonated solvent for ^{1}H spectra, or to the ^{13}C signal from the deuterated solvent. Chemical shift δ values for ^{1}H and ^{13}C spectra are reported in parts per million (ppm) relative to these referenced values, and multiplicities are abbreviated as s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. All ^{13}C NMR spectra were recorded with complete proton decoupling. FID files were processed using MestraNova 6.0.2 (MestreLab Research).

MS analyses: All high resolution mass spectrometry (HRMS) was performed on a Waters API Q-TOF Ultima spectrometer (Waters, Milford, MA, USA) at the MS unit of CAF.

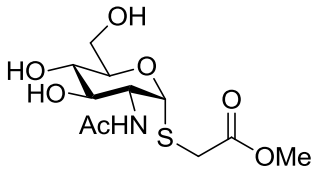
2. *Preparative scale biocatalytic reactions: Characterization data*



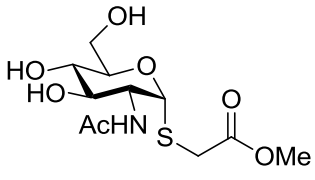
Phenyl 2-acetamido-2-deoxy-1-thio-α-D-glucopyranoside [Table 2, Entry 1; Reaction with CpGH89–E483A, 2NP-α-GlcNAc as donor and thiophenol as acceptor]. Obtained as light yellow solid (4.3 mg, 18%). TLC (silica gel, 15% MeOH in DCM): R_f = 0.41; ^{1}H NMR (600 MHz, D_2O) δ 7.48 (dd, *J* = 8.2, 1.2 Hz, 2H), 7.31–7.24 (m, 3H), 5.68 (d, *J* = 5.2 Hz, 1H), 4.10–4.06 (m, 2H), 3.80 (dd, *J* = 12.1, 2.5 Hz, 1H), 3.75 (dd, *J* = 12.1, 5.0 Hz, 1H), 3.65 (dd, *J* = 11.1, 8.7 Hz, 1H), 3.43 (dd, *J* = 9.8, 8.8 Hz, 1H), 2.00 (s, 3H). NMR data are in agreement with those previously published (Gammon, D.W. et al., 2010). HRMS (*m/z*): $[M+H]^+$ calcd. for $C_{14}H_{20}NO_5S$ 314.1062; found 314.1064.



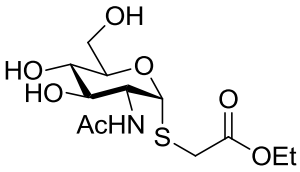
2,2,2-Trifluoroethyl 2-acetamido-2-deoxy-1-thio-α-D-glucopyranoside [Table 2, Entry 3; Reaction with CpGH89–E483A, 2NP-α-GlcNAc as donor and 2,2,2-trifluoroethanethiol as acceptor]. Obtained as a white solid (3.7 mg, 15%). TLC (silica gel, EtOAc/MeOH/H_2O, 7:2:1 v/v): R_f = 0.77; ^{1}H NMR (600 MHz, D_2O) δ 5.60 (d, *J* = 5.4 Hz, 1H), 4.13 (dd, *J* = 11.1, 5.4 Hz, 1H), 4.01 (ddd, *J* = 10.1, 5.1, 2.3 Hz, 1H), 3.88 (dd, *J* = 12.4, 2.3 Hz, 1H), 3.83 (dd, *J* = 12.5, 5.2 Hz, 1H), 3.71 (dd, *J* = 11.1, 8.9 Hz, 1H), 3.43–3.55 (m, 2H), 3.34 (dq, *J* = 15.7, 10.4 Hz, 1H), 2.06 (s, 3H). ^{13}C NMR (150 MHz, D_2O) δ 175.22, 84.52, 73.53, 71.14, 70.79, 61.02, 54.44, 32.24, 32.00, 22.41. HRMS (*m/z*): $[M+H]^+$ calcd. for $C_{10}H_{17}F_3NO_5S$ 320.0780; found 320.0780.



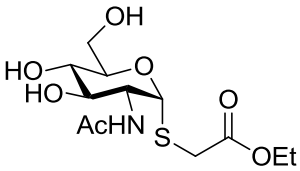
Methyl *S*-(2-acetamido-2-deoxy-α-D-glucopyranosyl) 2-thioacetate [Table 2, Entry 5; Reaction with CpGH89–E483A, 2NP-α-GlcNAc as donor and methyl thioglycolate as acceptor]. Obtained as a white solid (19.6 mg, 83%). TLC (silica gel, EtOAc/MeOH/H_2O, 7:2:1 v/v): R_f = 0.54; ^{1}H NMR (400 MHz, D_2O) δ 5.55 (d, *J* = 5.4 Hz, 1H), 4.11 (dd, *J* = 11.0, 5.4 Hz, 1H), 3.96–4.01 (m, 1H), 3.82–3.84 (m, 2H), 3.76 (s, 3H), 3.67–3.74 (m, 1H), 3.56 (d, *J* = 15.7 Hz, 1H), 3.53 (dd, *J* = 12.2, 6.7 Hz, 1H), 3.42 (d, *J* = 15.7 Hz, 1H), 2.06 (s, 3H). ^{13}C NMR (100 MHz, D_2O) δ 175.07, 173.87, 84.56, 73.40, 71.29, 70.73, 60.88, 54.39, 53.77, 32.50, 22.45. HRMS (*m/z*): $[M-H]^-$ calcd. for $C_{11}H_{18}NO_7S$ 308.0804; found 308.0797.



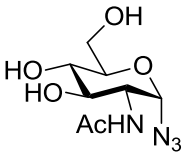
Methyl *S*-(2-acetamido-2-deoxy-α-D-glucopyranosyl) 2-thioacetate [Table 2, Entry 6; Reaction with CpGH89–E483S, 2NP-α-GlcNAc as donor and methyl thioglycolate as acceptor]. Obtained as white solid (11.5 mg, 49%). Characterization data as above.



Ethyl *S*-(2-acetamido-2-deoxy-α-D-glucopyranosyl) 2-thioacetate [Table 2, Entry 7; Reaction with CpGH89–E483A, 2NP-α-GlcNAc as donor and ethyl thioglycolate as acceptor]. Obtained as white solid (11.1 mg, 46%). TLC (silica gel, EtOAc/MeOH/H_2O, 7:2:1 v/v): R_f = 0.62; ^{1}H NMR (400 MHz, D_2O) δ 5.56 (d, *J* = 5.4 Hz, 1H), 4.22 (q, *J* = 7.2 Hz, 2H), 4.11 (dd, *J* = 11.0, 5.4 Hz, 1H), 3.97–4.02 (m, 1H), 3.81–3.85 (m, 2H), 3.67–3.74 (m, 1H), 3.49–3.56 (m, 2H), 3.35–3.42 (m, 1H), 2.06 (s, 3H), 1.28 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (100 MHz, D_2O) δ 176.98, 175.32, 86.37, 75.34, 73.23, 72.68, 65.44, 62.84, 56.33, 34.49, 24.38, 15.81. HRMS (*m/z*): $[M-H]^-$ calcd. for $C_{12}H_{20}NO_7S$ 322.0960; found 322.0948.



Ethyl *S*-(2-acetamido-2-deoxy-α-D-glucopyranosyl) 2-thioacetate [Table 2, Entry 8; Reaction with CpGH89–E483S, 2NP-α-GlcNAc as donor and ethyl thioglycolate as acceptor]. Obtained as white solid (8.0 mg, 33%). Characterization data as above.

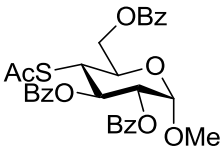


2-Acetamido-2-deoxy-α-D-glucopyranosyl azide [Table 2, Entry 15; Reaction with CpGH89–E483A, 2NP-α-GlcNAc as donor and sodium azide as acceptor]. Obtained as white solid (14.7 mg, 79%). TLC (silica gel, EtOAc/MeOH/H_2O, 7:2:1 v/v): R_f = 0.59; ^{1}H NMR (300 MHz, CD_3OD) δ 5.47 (d, *J* = 4.1 Hz, 1H), 3.92 (dd, *J* = 10.6, 4.2 Hz, 1H), 3.84–3.88 (m 1H), 3.65–3.75 (m, 2H), 3.56 (dd, *J* = 10.6, 8.8 Hz, 1H), 3.33–3.41 (m, 1H), 2.00 (s, 3H). ^{13}C NMR (75 MHz, CD_3OD) δ 89.98, 76.11, 72.09, 71.80, 62.39, 54.81, 22.46. HRMS (*m/z*): $[M-H]^-$ calcd. for $C_8H_{13}N_4O_5$ 245.0886; found 245.0879.

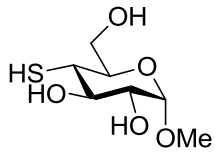
3. *Chemical syntheses:*

3.1 Synthesis of methyl 4-thio-α-D-glucopyranoside

Methyl 4-thio-α-D-glucopyranoside was synthesized by modification of a previously reported method (Chen et al., 2010) as described below.



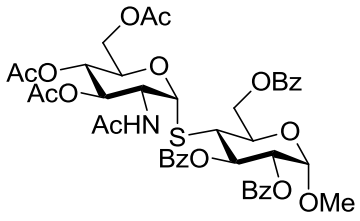
Methyl 4-*S*-acetyl-2,3,6-tri-*O*-benzoyl-4-thio-α-D-glucopyranoside. Under a stream of argon, Tf_2O (1.93 mL, 11.50 mmol, 2.3 equiv) was added in three portions to a solution of methyl 2,3,6-tri-*O*-benzoyl-α-D-galactopyranoside (2.50 g, 4.94 mmol) (Carbosynth) in dry DCM (42 mL) and dry pyridine (9.7 mL) at 0°C. After 1 h of stirring the reaction mixture under these conditions, 75 mL of DCM was added. The mixture was washed sequentially with 50 mL of ice cold 1M HCl, 50 mL of cold saturated $NaHCO_3$ and 50 mL of cold brine, dried over $MgSO_4$ and the solvent evaporated under diminished pressure to give a yellow residue. The resulting residue was purified by flash column chromatography (4:1 hexane/EtOAc) to give the triflate (1.43g, 45%) as a white powder. To the triflate (0.90 g, 1.40 mmol) was added 10 mL DMPU (1,3-dimethyl-3,4,5,6-tetra-hydro-2(1*H*)-pyrimidinone) and KSAc (0.48 g, 4.20 mmol, 3.0 equiv), and the suspension was stirred at room temperature for 1.5 h. The reaction mixture was diluted with 100 mL of a 1:1 mixture of *n*-hexane and EtOAc, subsequently washed with water (5×50 mL) and brine (50 mL), and dried over $MgSO_4$. The solvent was evaporated under diminished pressure to give a yellow residue, which was purified by flash column chromatography (4:1 and 2:1 hexane/EtOAc) to give the product (0.51 g, 65%) as a white powder. R_f 0.53 (2:1 Hexane/EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.09–8.16 (m, 2H), 7.91–8.00 (m, 4H), 7.56–7.63 (m, 1H), 7.49 (ddd, *J* = 4.7, 3.5, 3.0 Hz, 4H), 7.36 (td, *J* = 7.8, 2.7 Hz, 4H), 5.94 (dd, *J* = 10.9, 9.6 Hz, 1H), 5.20–5.29 (m, 2H), 4.65 (dd, *J* = 12.1, 2.2 Hz, 1H), 4.56 (dd, *J* = 12.1, 5.1 Hz, 1H), 4.29 (ddd, *J* = 11.6, 5.0, 2.0 Hz, 1H), 4.09–4.22 (m, 1H), 3.44 (s, 3H), 2.21 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.64, 169.64, 166.40, 165.92, 165.84, 133.44, 133.34, 133.30, 130.04, 129.91, 129.89, 129.37, 129.20, 128.59, 128.51, 128.49, 97.36, 97.13, 73.26, 72.04, 70.71, 69.41, 68.85, 68.73, 67.64, 63.98, 55.71, 44.28, 30.79, 20.73. ESI-MS (*m/z*): $[M+Na]^+$ calcd. for $C_{30}H_{32}NO_9SNa$ 587.1346; found 582.1351.



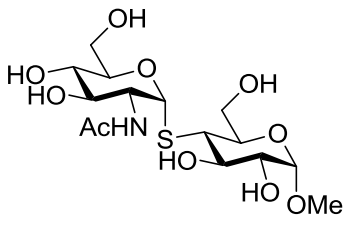
Methyl 4-thio-α-D-glucopyranoside. Argon was bubbled through a suspension of the thioacetate (0.50 g, 0.886 mmol) in 18 mL of dry methanol for 0.5 h at room temperature, after which sodium methoxide solution (0.25 mL, 5.4 M, 2.57 mmol, 2.9 equiv) was added. The reaction mixture was left stirring at room temperature under argon for 48 h, followed by addition of IR-120(H^+) ion exchange resin (0.50 g). The resin-containing mixture was stirred for 0.5 h at room temperature, after which the resin was filtered and washed with dry methanol. The combined filtrates were concentrated in vacuo until 3 mL of solvent remained. A solution of DTT (0.36 g in 15 mL distilled water) was added to the remaining solvent while stirring. Argon was bubbled through the reaction mixture for 0.5 h, which was then left stirring under argon overnight. The solvent was evaporated under reduced pressure to give a white residue, which was purified by flash column chromatography (15:1 and 9.1, DCM: CH_3OH) to give the product as a white powder (170 mg, 91%). R_f 0.14 (15:1 DCM: CH_3OH). ^{1}H NMR (400 MHz, CD_3OD) δ 4.73 (d, *J* = 4.7 Hz, 1H), 3.81 (dd, *J* = 12.0, 3.5 Hz, 2H), 3.58 (ddd, *J* = 10.8, 4.6, 2.4 Hz, 1H), 3.51 (dd, *J* = 12.6, 6.8 Hz, 1H), 3.43 – 3.37 (m, 4H), 2.72 (t, *J* = 10.4 Hz, 1H). ^{13}C NMR (100 MHz, CD_3OD) δ 101.35, 75.51, 74.97, 74.33, 63.12, 55.56, 43.37. ESI-MS (*m/z*): $[M-H]^-$ calcd. for $C_7H_{13}O_5S$ 209.0484; found 209.0485.

3.2 Synthesis of methyl 4-S-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-4-thio-α-D-glucopyranoside.

The title compound was synthesized by modification of the method reported above, using 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-1-thio-α-D-glucopyranose instead of thioacetate as nucleophile in the displacement reaction.



Methyl 4-*S*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-gluco-pyranosyl)-4-thio-3,4,6-tri-*O*-benzoyl-α-D-glucopyranoside. A solution of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-1-thio-α-D-glucopyranose (0.563 g, 1.54 mmol) (Knapp, S. et al., 2001) in 3 mL of dry *N*,*N*-dimethylformamide (DMF) was treated with 0.058 g (1.53 mmol) of sodium borohydride while stirring under nitrogen. After 30 minutes, the triflate prepared as described above (1.53 g, 2.31 mmol, 1.5 equiv) was added and the reaction was stirred at room temperature for 4 h. The reaction mixture was neutralized by addition of glacial acetic acid (44 µL), followed by dilution with 50 µL of ethyl acetate. The reaction mixture was washed with water (4×50 mL) and dried over Na_2SO_4. The solvent was removed under diminished pressure to give a yellowish residue that was purified by flash column chromatography (hexane/EtOAc/acetone, 4:3:1) to give the product (0.315 g, 24%) as a white powder. R_f 0.35 (5% MeOH in DCM). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08–8.11 (m, 2H), 7.89–7.96 (m, 4H), 7.61 (t, *J* = 7.4 Hz, 1H), 7.51 (dt, *J* = 11.4, 7.4 Hz, 4H), 7.36 (dt, *J* = 11.6, 7.8 Hz, 4H), 6.04 (t, *J* = 10.4 Hz, 1H), 5.54 (d, *J* = 5.3 Hz, 1H), 5.44 (d, *J* = 9.5 Hz, 1H), 5.19 (d, *J* = 3.5 Hz, 1H), 5.06–5.15 (m, 2H), 4.87 (ddd, *J* = 11.9, 9.1, 5.8 Hz, 2H), 4.60 (dd, *J* = 11.9, 5.8 Hz, 1H), 4.47 (ddd, *J* = 11.2, 9.6, 5.3 Hz, 1H), 4.18–4.33 (m, 3H), 4.04 (d, *J* = 10.4 Hz, 1H), 3.44 (s, 3H), 3.30 (t, *J* = 11.0 Hz, 1H), 2.08 (s, 3H), 1.99 (s, 3H), 1.93 (s, 3H), 1.61 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.28, 170.70, 169.53, 169.24, 166.32, 165.93, 165.64, 133.85, 133.53, 130.01, 129.87, 129.78, 129.69, 129.01, 128.89, 128.85, 128.74, 128.53, 97.2, 86.82, 73.27, 72.06, 71.30, 69.72, 68.89, 67.69, 64.25, 61.67, 55.82, 51.76, 47.91, 22.48, 20.83, 20.70, 20.68. ESI-MS (*m/z*): $[M+H]^+$ calcd. for $C_{42}H_{46}NO_6S$ 852.2537; found 852.2549.



Methyl 4-*S*-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-4-thio-α-D-gluco-pyranoside. To a solution of methyl 4-*S*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl)-4-thio-3,4,6-tri-*O*-benzoyl-α-D-glucopyranoside (0.315 g, 0.370 mmol) in 5 mL of dry methanol was added sodium methoxide solution (0.770 g in 5 mL dry methanol) and the mixture left to stir at room temperature overnight (Bengtsson, M. et al, 1998). This was followed by neutralization by addition of 0.5 g of IR-120(H^+) ion exchange resin, which was stirred for 0.5h at room temperature. The resin was filtered and washed with dry methanol followed by removal of the solvent from the combined filtrates in vacuo. The resulting residue was purified using flash column chromatography (61:33:6, DCM/MeOH/H_2O) to give the product as a white powder (70 mg, 46%). R_f 0.42 (61:33:6, DCM/MeOH/H_2O). ^{1}H NMR (600 MHz, D_2O) δ 5.66 (d, *J* = 5.4 Hz, 1H), 4.87 (d, *J* = 3.7 Hz, 1H), 4.12 – 4.05 (m, 2H), 3.97 (dd, *J* = 12.1, 2.2 Hz, 1H), 3.94 – 3.89 (m, 2H), 3.89 – 3.78 (m, 3H), 3.64 (dd, *J* = 11.1, 8.8 Hz, 1H), 3.57 (dd, *J* = 9.5, 3.7 Hz, 1H), 3.49 (dd, *J* = 9.8, 9.0 Hz, 1H), 3.43 (s, 3H), 3.37 (s, 1H), 2.79 (t, *J* = 10.9 Hz, 1H), 2.08 (s, 3H). ^{13}C NMR (150 MHz, D_2O) δ 174.94, 100.04, 84.30, 73.84, 73.07, 72.21, 71.50, 71.45, 70.85, 62.08, 61.09, 55.71, 54.75, 48.22, 22.46. ESI-MS (*m/z*): $[M+H]^+$ calcd. for $C_{15}H_{28}NO_{10}S$ 414.1434; found 414.1426.

SUPPLEMENTARY REFERENCES

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Knapp, S. and Myers, D. S. (2001) α-GlcNAc Thioconjugates. *J. Org. Chem.* **66**, 3636–3638.

ORIGINAL SPECTRA

(Following pages)

Original spectra of compounds prepared by preparative scale biocatalysis (in order):

^{1}H NMR spectrum of phenyl 2-acetamido-2-deoxy-1-thio-α-D-glucopyranoside.
ESI-TOF (positive mode) spectrum of phenyl 2-acetamido-2-deoxy-1-thio-α-D-glucopyranoside.

^{1}H NMR spectrum of 2,2,2-trifluoroethyl 2-acetamido-2-deoxy-1-thio-α-D-glucopyranoside.
^{13}C NMR spectrum of 2,2,2-trifluoroethyl 2-acetamido-2-deoxy-1-thio-α-D-glucopyranoside.
ESI-TOF (positive mode) spectrum of 2,2,2-trifluoroethyl 2-acetamido-2-deoxy-1-thio-α-D-glucopyrano-side.

^{1}H NMR spectrum of methyl *S*-(2-acetamido-2-deoxy-α-D-glucopyranosyl) 2-thioacetate.
^{13}C NMR spectrum of methyl *S*-(2-acetamido-2-deoxy-α-D-glucopyranosyl) 2-thioacetate.
ESI-TOF (negative mode) spectrum of methyl *S*-(2-acetamido-2-deoxy-α-D-glucopyranosyl) 2-thioacetate.

^{1}H NMR spectrum of ethyl *S*-(2-acetamido-2-deoxy-α-D-glucopyranosyl) 2-thioacetate.
^{13}C NMR spectrum of ethyl *S*-(2-acetamido-2-deoxy-α-D-glucopyranosyl) 2-thioacetate.
ESI-TOF (negative mode) spectrum of ethyl *S*-(2-acetamido-2-deoxy-α-D-glucopyranosyl) 2-thioacetate.

^{1}H NMR spectrum of 2-acetamido-2-deoxy-α-D-glucopyranosyl azide.
^{13}C NMR spectrum of 2-acetamido-2-deoxy-α-D-glucopyranosyl azide.
ESI-TOF (negative mode) spectrum of 2-acetamido-2-deoxy-α-D-glucopyranosyl azide.

Original spectra of compounds prepared by chemical synthesis (in order):

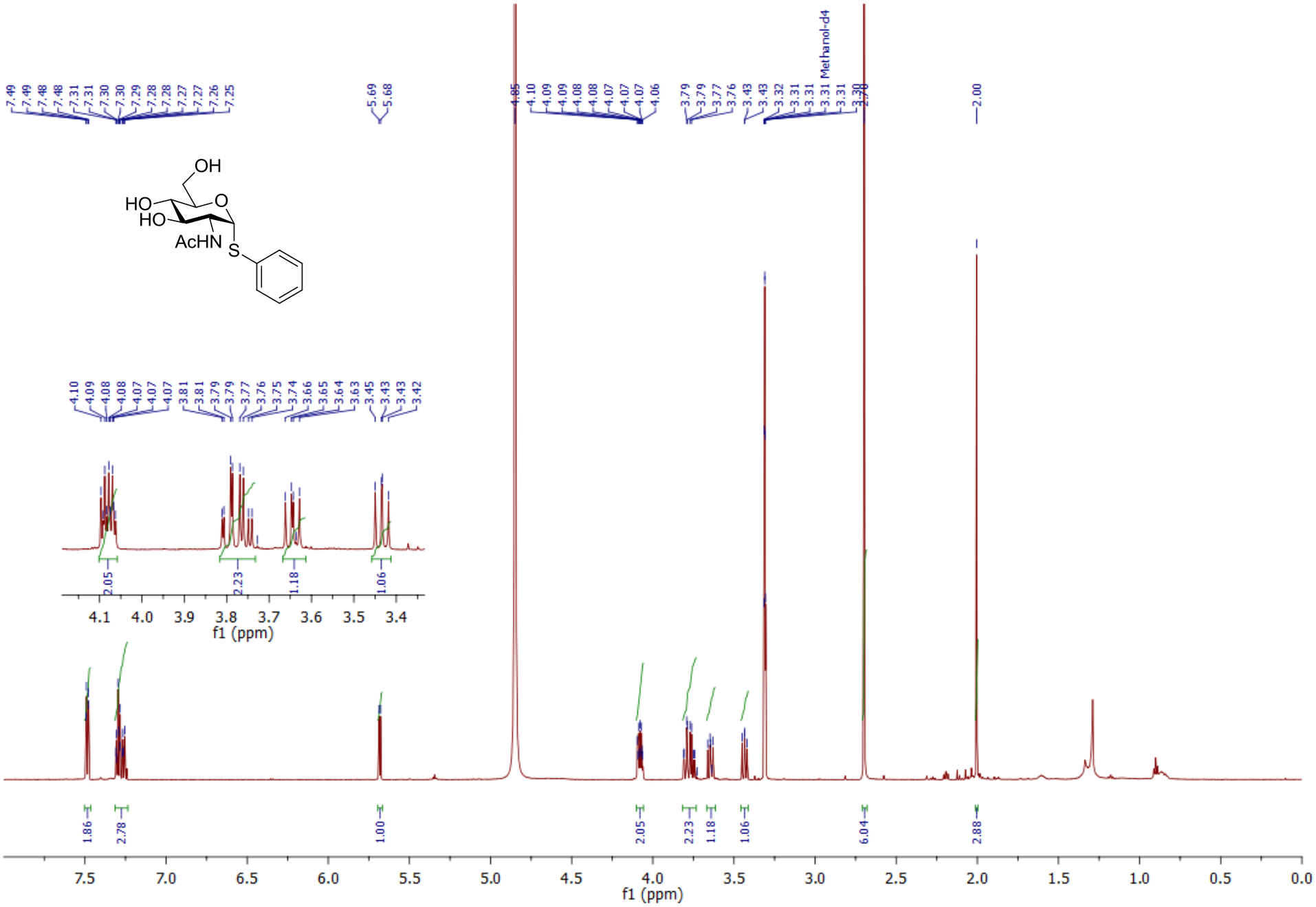
^{1}H NMR spectrum of methyl 4-*S*-acetyl-2,3,6-tri-*O*-benzoyl-4-thio-α-D-glucopyranoside.
^{13}C NMR spectrum of methyl 4-*S*-acetyl-2,3,6-tri-*O*-benzoyl-4-thio-α-D-glucopyranoside.
ESI-TOF (positive mode) spectrum of methyl 4-*S*-acetyl-2,3,6-tri-*O*-benzoyl-4-thio-α-D-glucopyranoside.

^{1}H NMR spectrum of methyl 4-thio-α-D-glucopyranoside.
^{13}C NMR spectrum of methyl 4-thio-α-D-glucopyranoside.
ESI-TOF (negative mode) spectrum of methyl 4-thio-α-D-glucopyranoside.

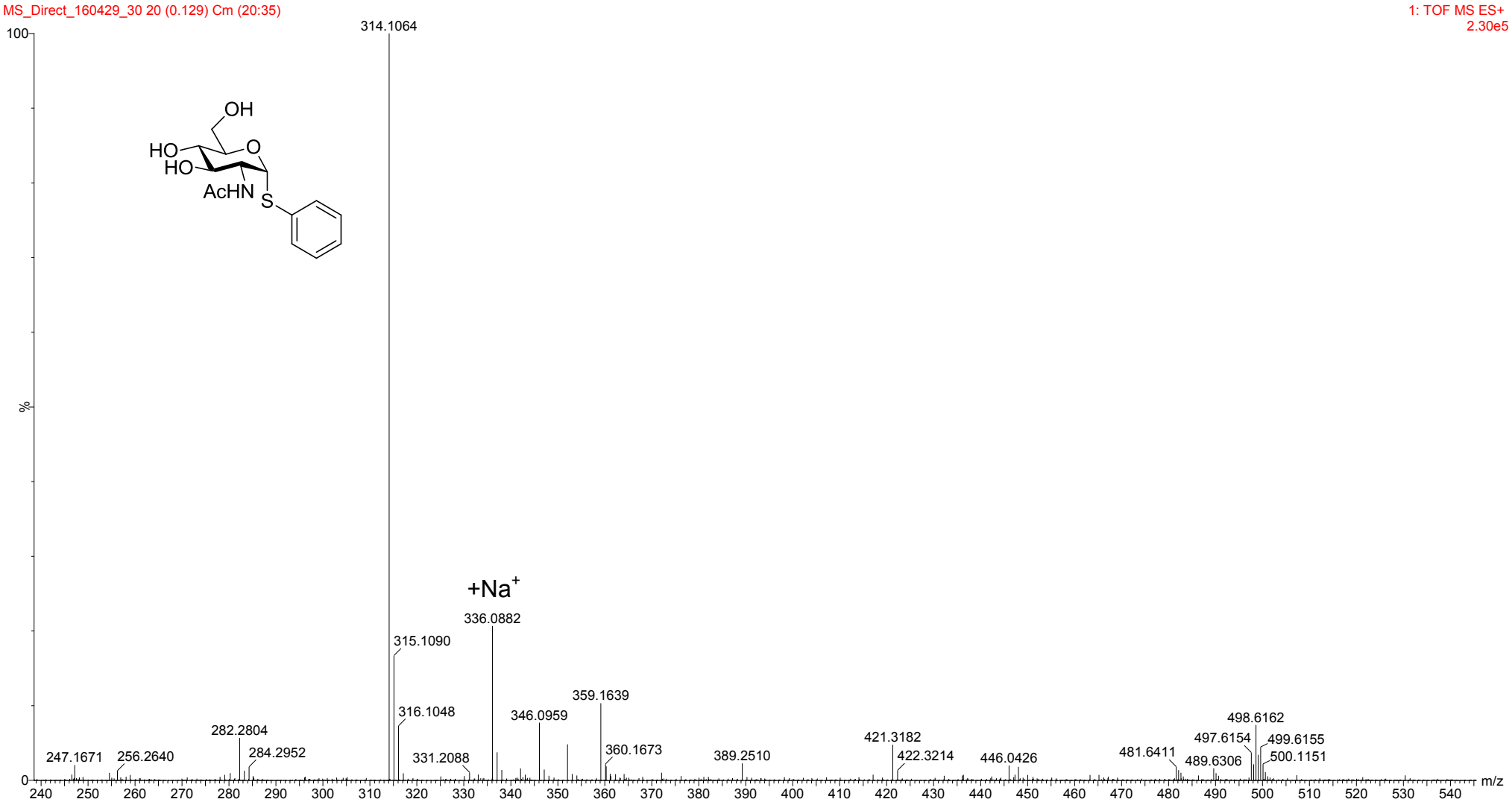
^{1}H NMR spectrum of methyl 4-*S*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-gluco-pyranosyl)-4-thio-3,4,6-tri-*O*-benzoyl-α-D-glucopyranoside.
^{13}C NMR spectrum of methyl 4-*S*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-gluco-pyranosyl)-4-thio-3,4,6-tri-*O*-benzoyl-α-D-glucopyranoside.
ESI-TOF (positive mode) spectrum of methyl 4-*S*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-gluco-pyranosyl)-4-thio-3,4,6-tri-*O*-benzoyl-α-D-glucopyranoside.

^{1}H NMR spectrum of methyl 4-*S*-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-4-thio-α-D-glucopyranoside.
^{13}C NMR spectrum of methyl 4-*S*-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-4-thio-α-D-glucopyranoside.
ESI-TOF (positive mode) spectrum of methyl 4-*S*-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-4-thio-α-D-glucopyranoside.

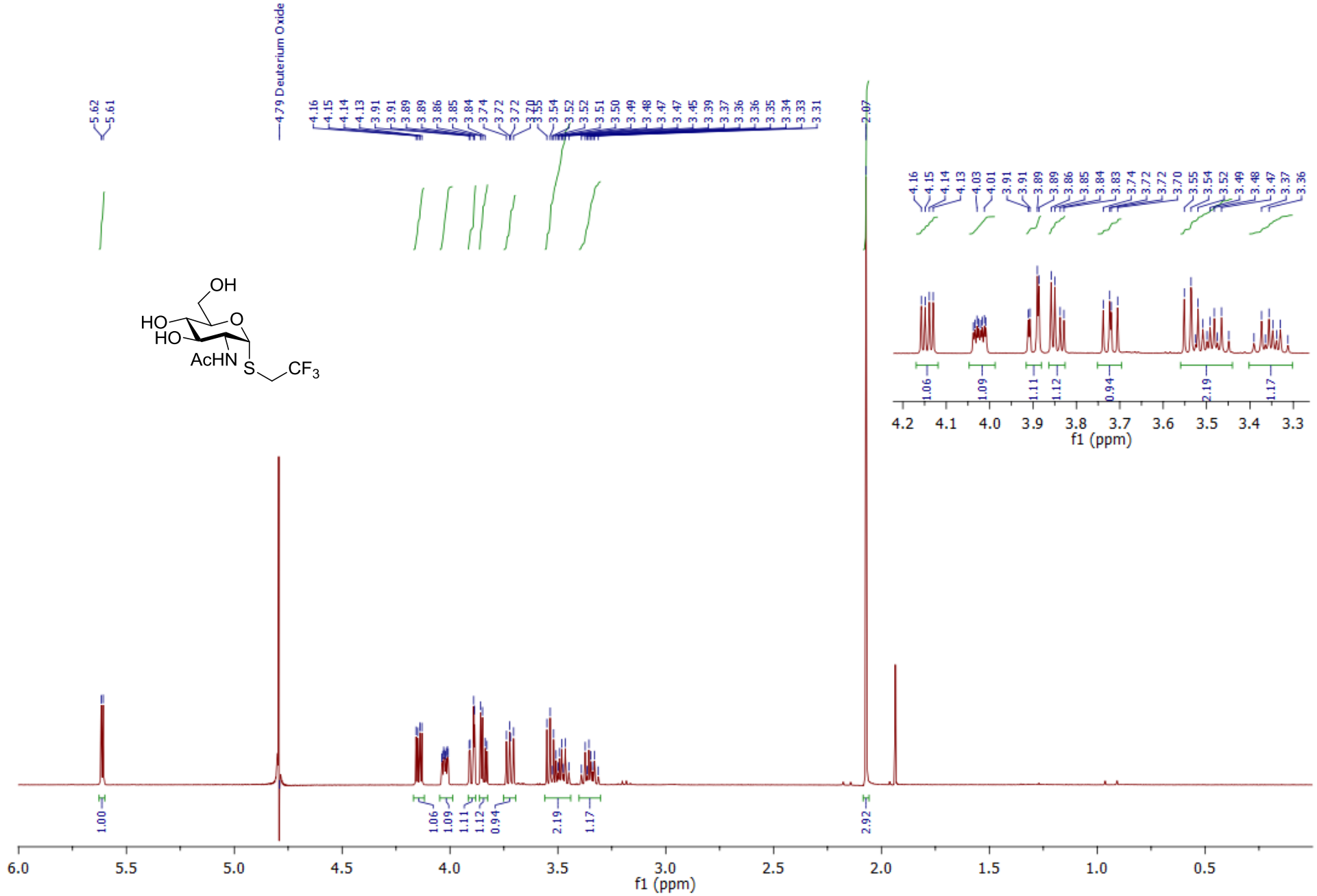
Phenyl 2-acetamido-2-deoxy-1-thio-α-D-glucopyranoside: ^{1}H NMR spectrum (600 MHz)



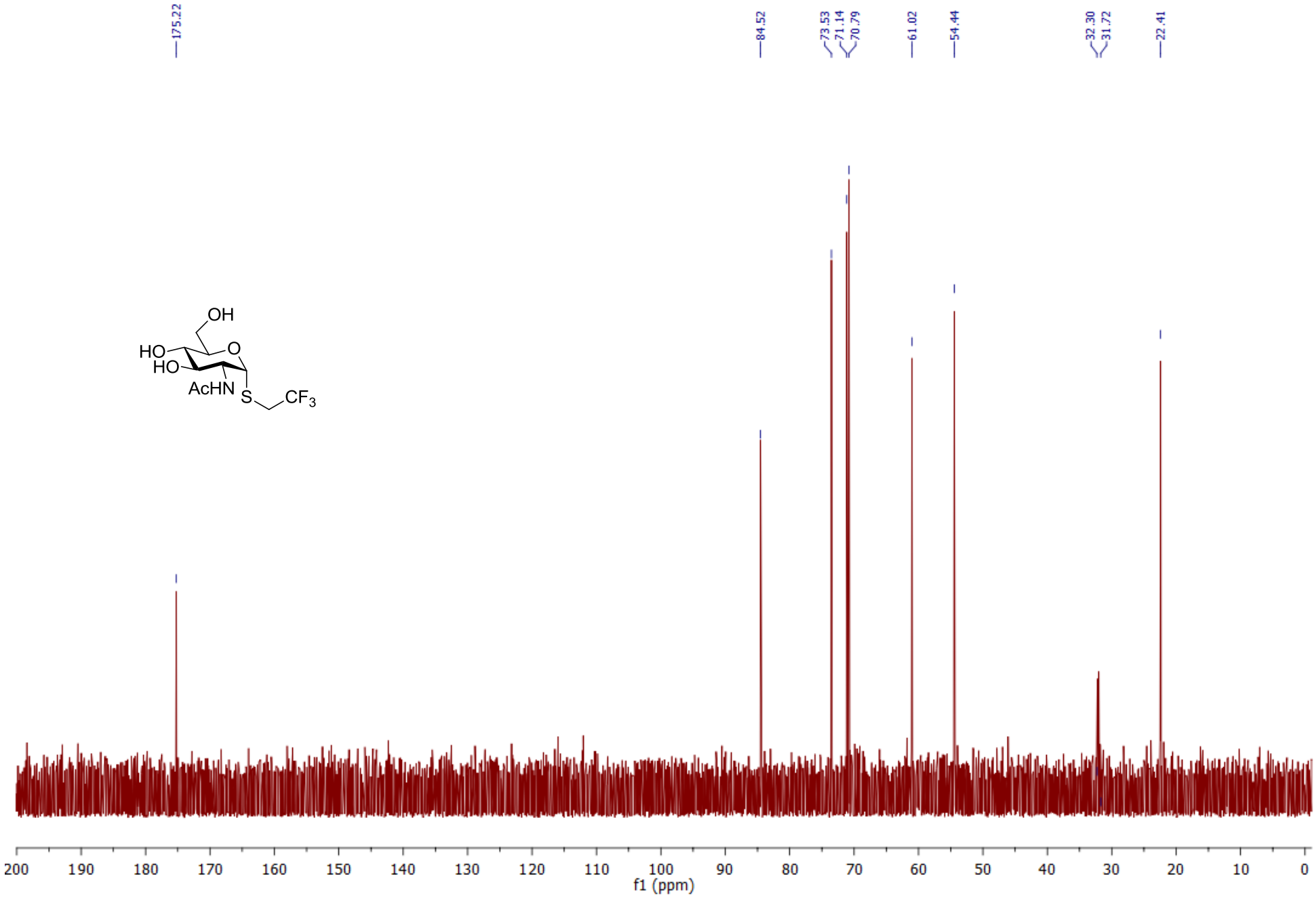
Phenyl 2-acetamido-2-deoxy-1-thio-α-D-glucopyranoside: mass spectrum (ESI-TOF+)



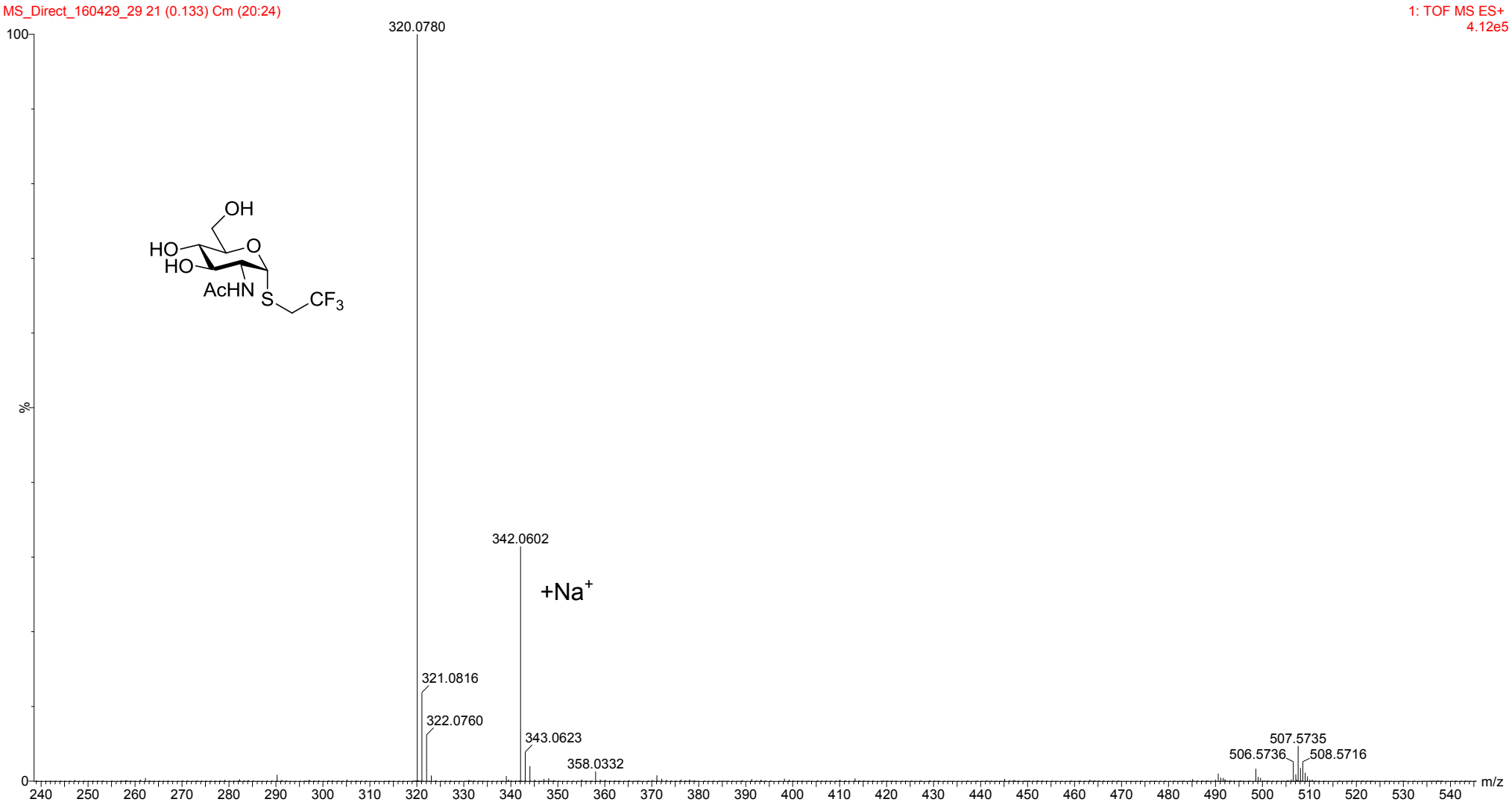
2,2,2-Trifluoroethyl 2-acetamido-2-deoxy-1-thio-α-D-glucopyranoside: ^{1}H NMR spectrum (600 MHz)



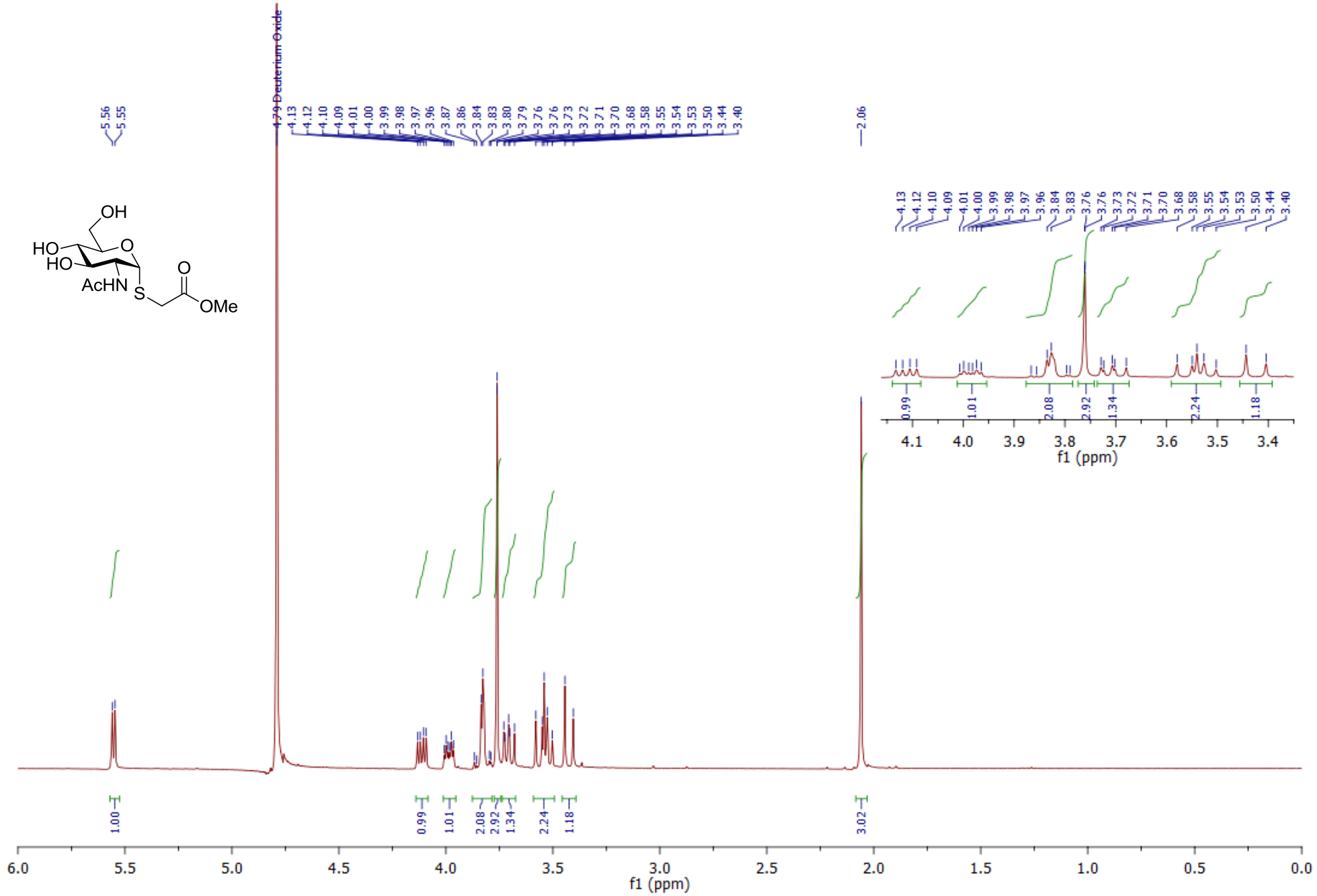
2,2,2-Trifluoroethyl 2-acetamido-2-deoxy-1-thio-α-D-glucopyranoside: ^{13}C NMR spectrum (150 MHz)



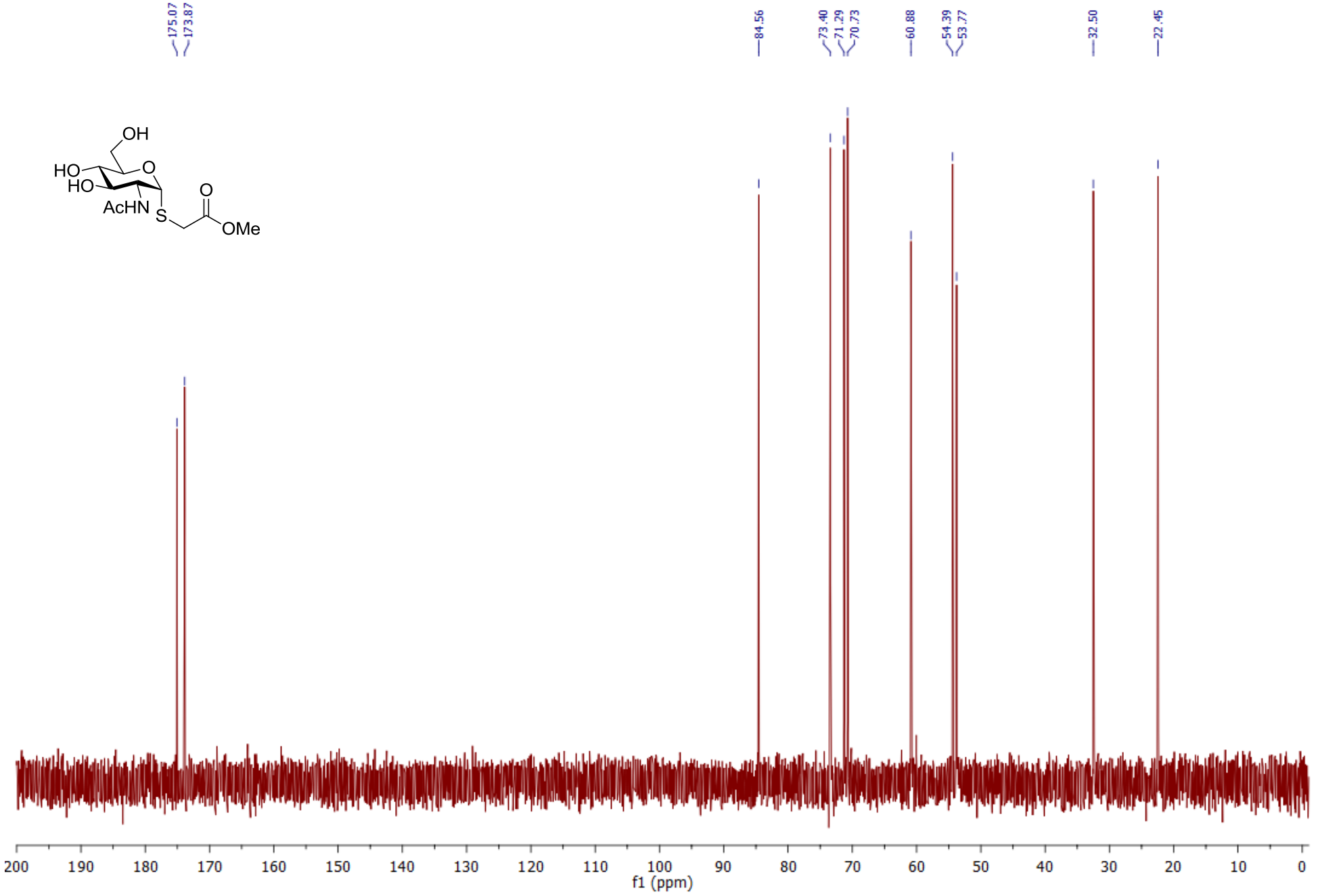
2,2,2-Trifluoroethyl 2-acetamido-2-deoxy-1-thio-α-D-glucopyranoside: mass spectrum (ESI-TOF+)



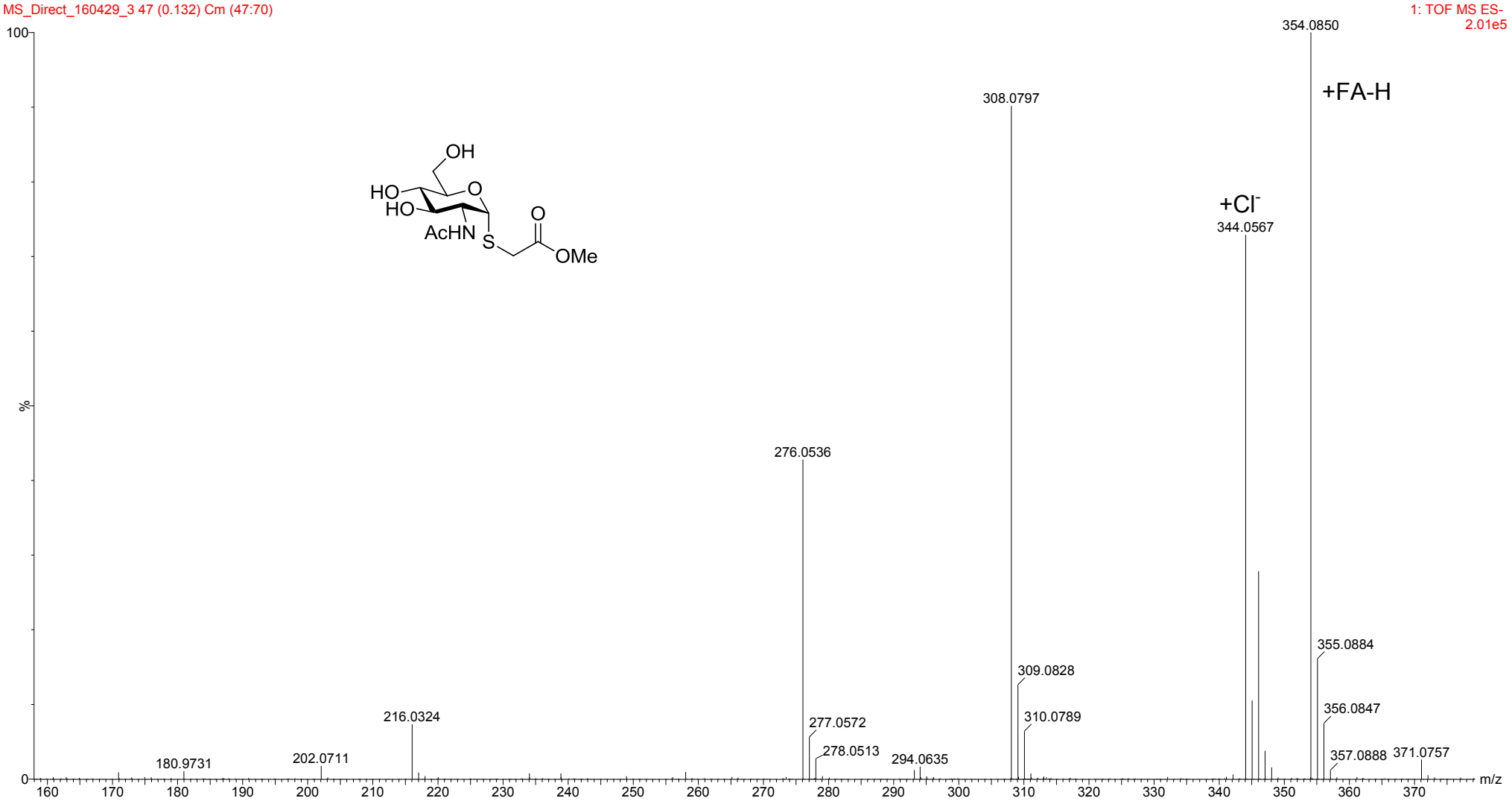
Methyl *S*-(2-acetamido-2-deoxy-α-D-glucopyranosyl) 2-thioacetate: ^{1}H NMR spectrum (400 MHz)



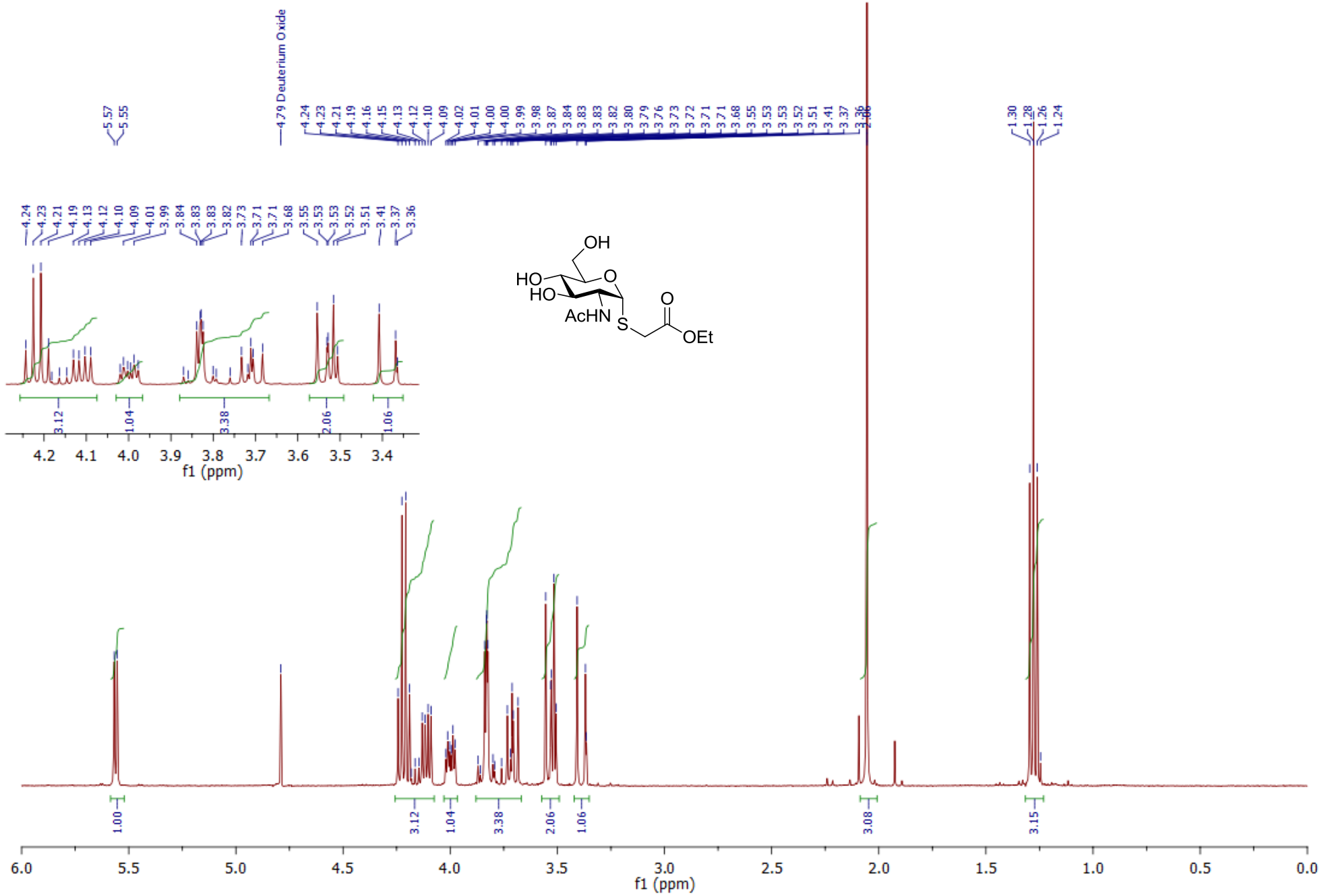
Methyl *S*-(2-acetamido-2-deoxy-α-D-glucopyranosyl) 2-thioacetate: ^{13}C NMR spectrum (100 MHz)



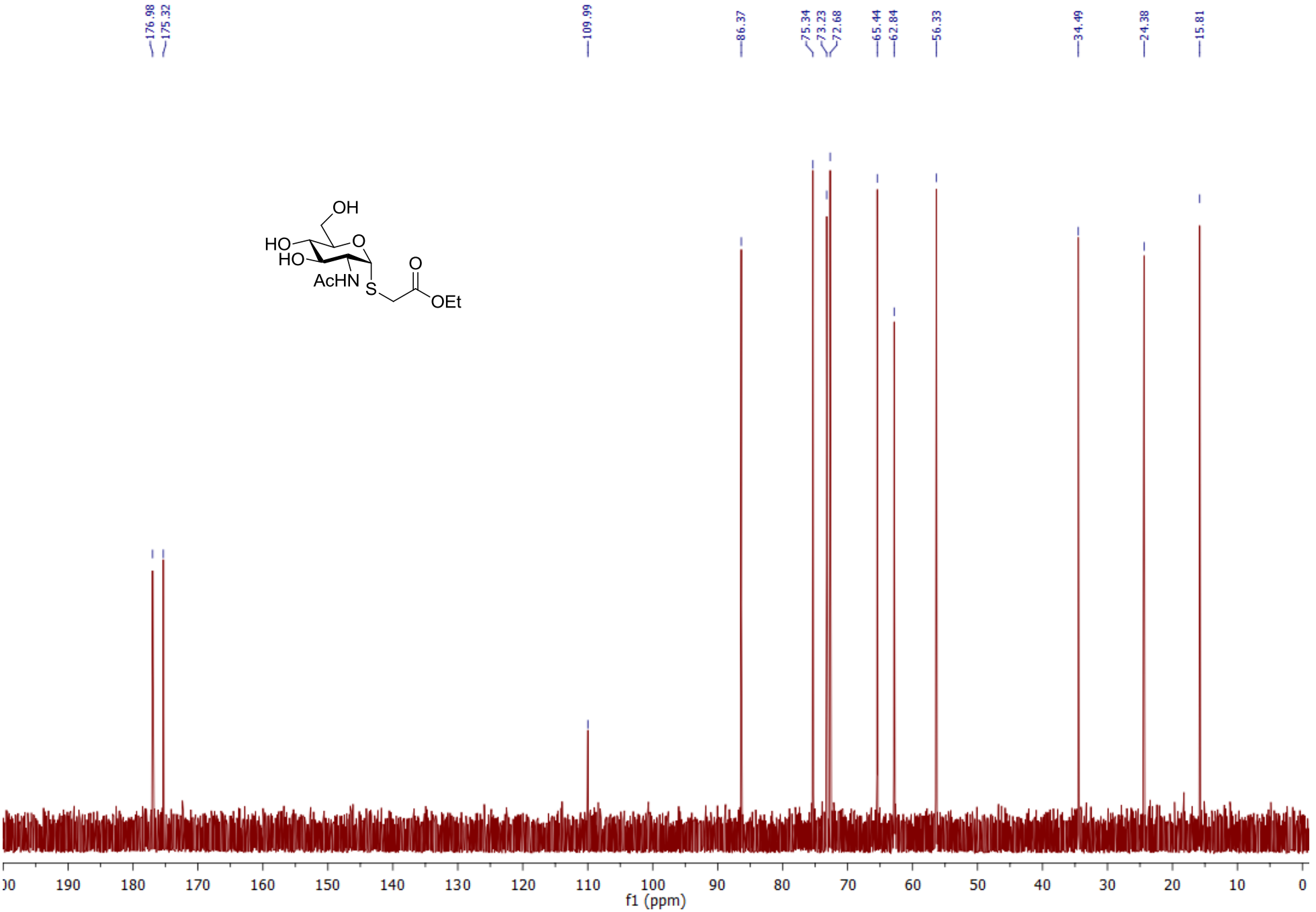
Methyl *S*-(2-acetamido-2-deoxy-α-D-glucopyranosyl) 2-thioacetate: mass spectrum (ESI-TOF-)



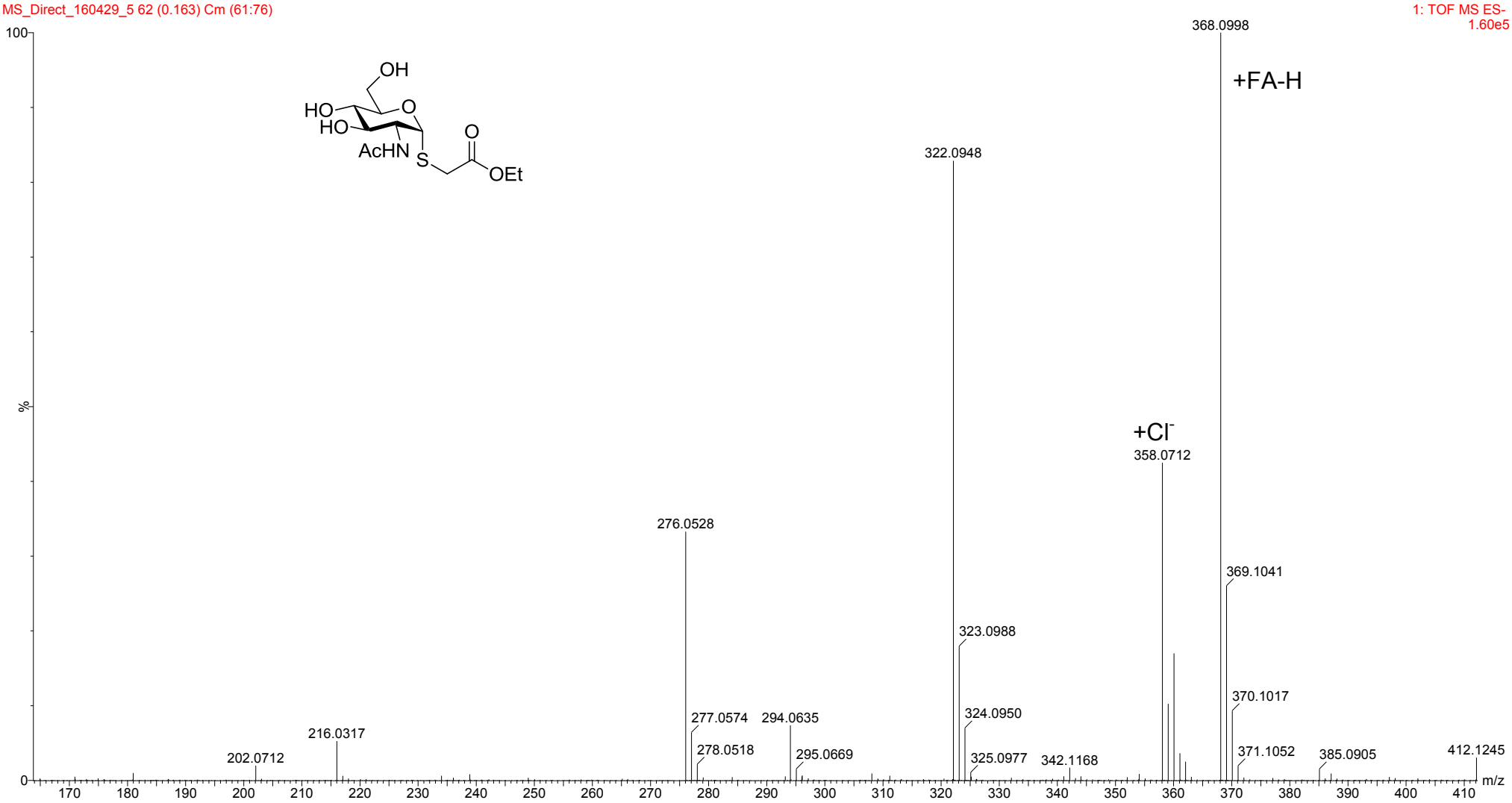
Ethyl *S*-(2-acetamido-2-deoxy-α-D-glucopyranosyl) 2-thioacetate: ^{1}H NMR spectrum (400 MHz)



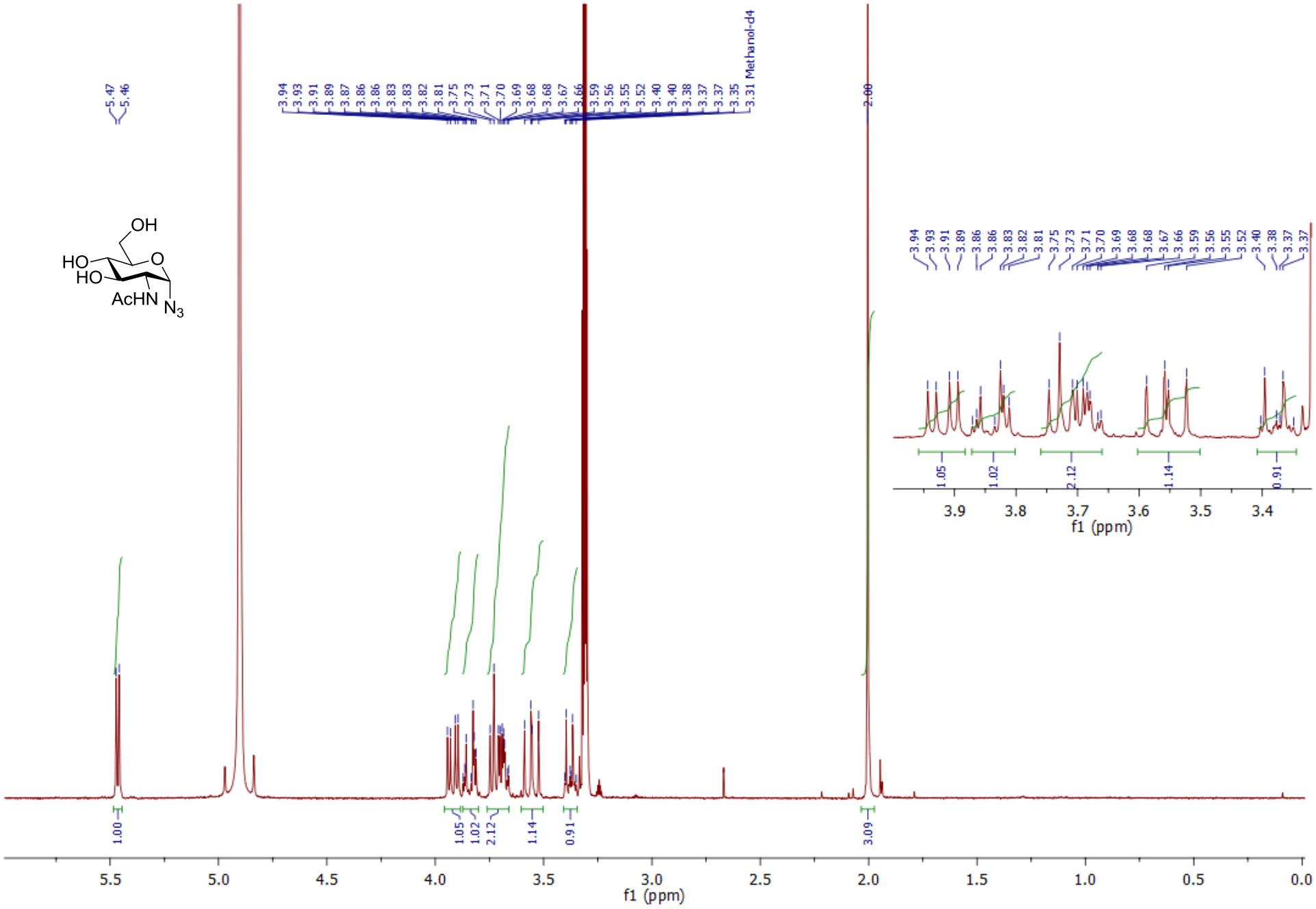
Ethyl *S*-(2-acetamido-2-deoxy-α-D-glucopyranosyl) 2-thioacetate: ^{13}C NMR spectrum (100 MHz)



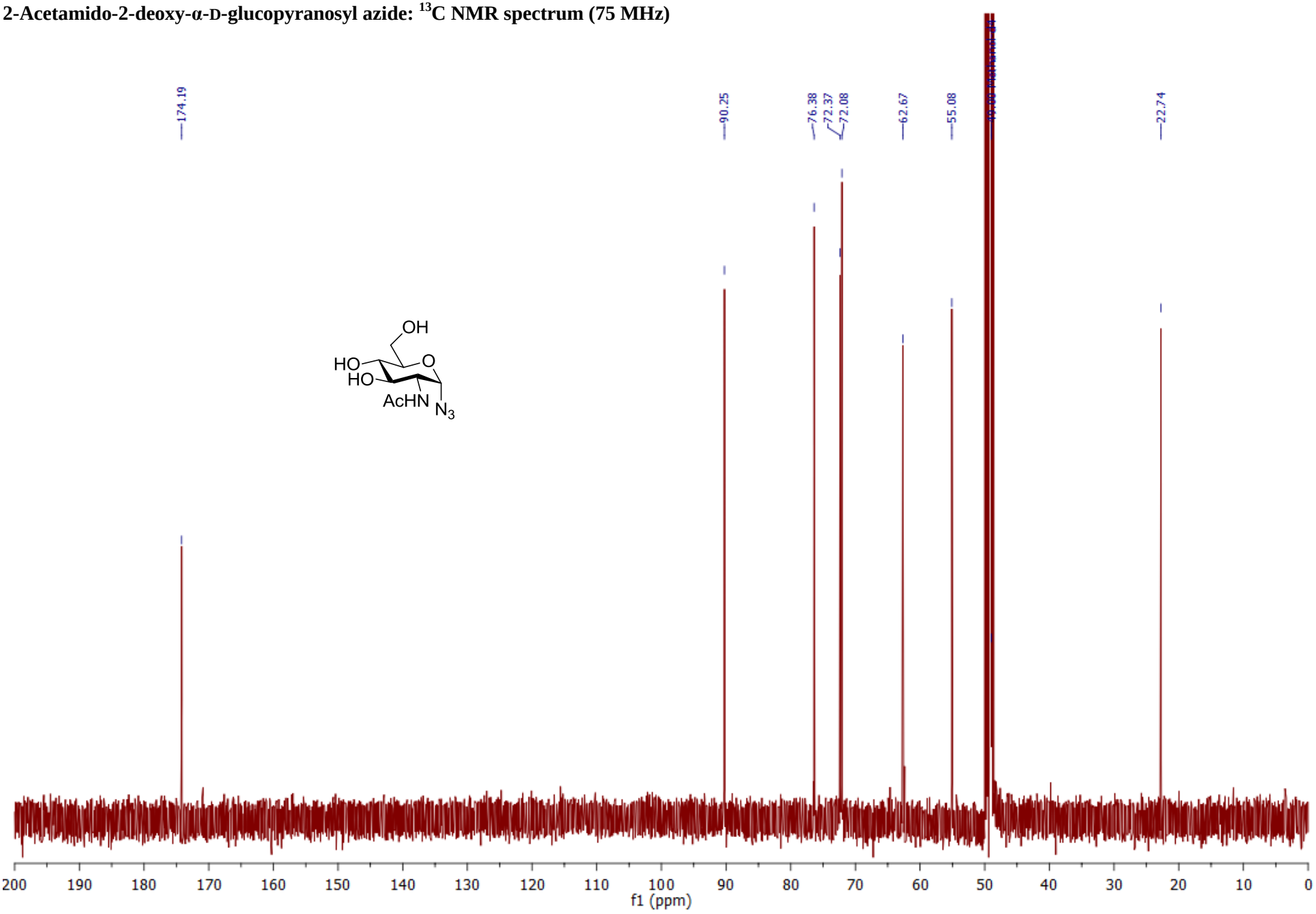
Ethyl *S*-(2-acetamido-2-deoxy-α-D-glucopyranosyl) 2-thioacetate: mass spectrum (ESI-TOF-)



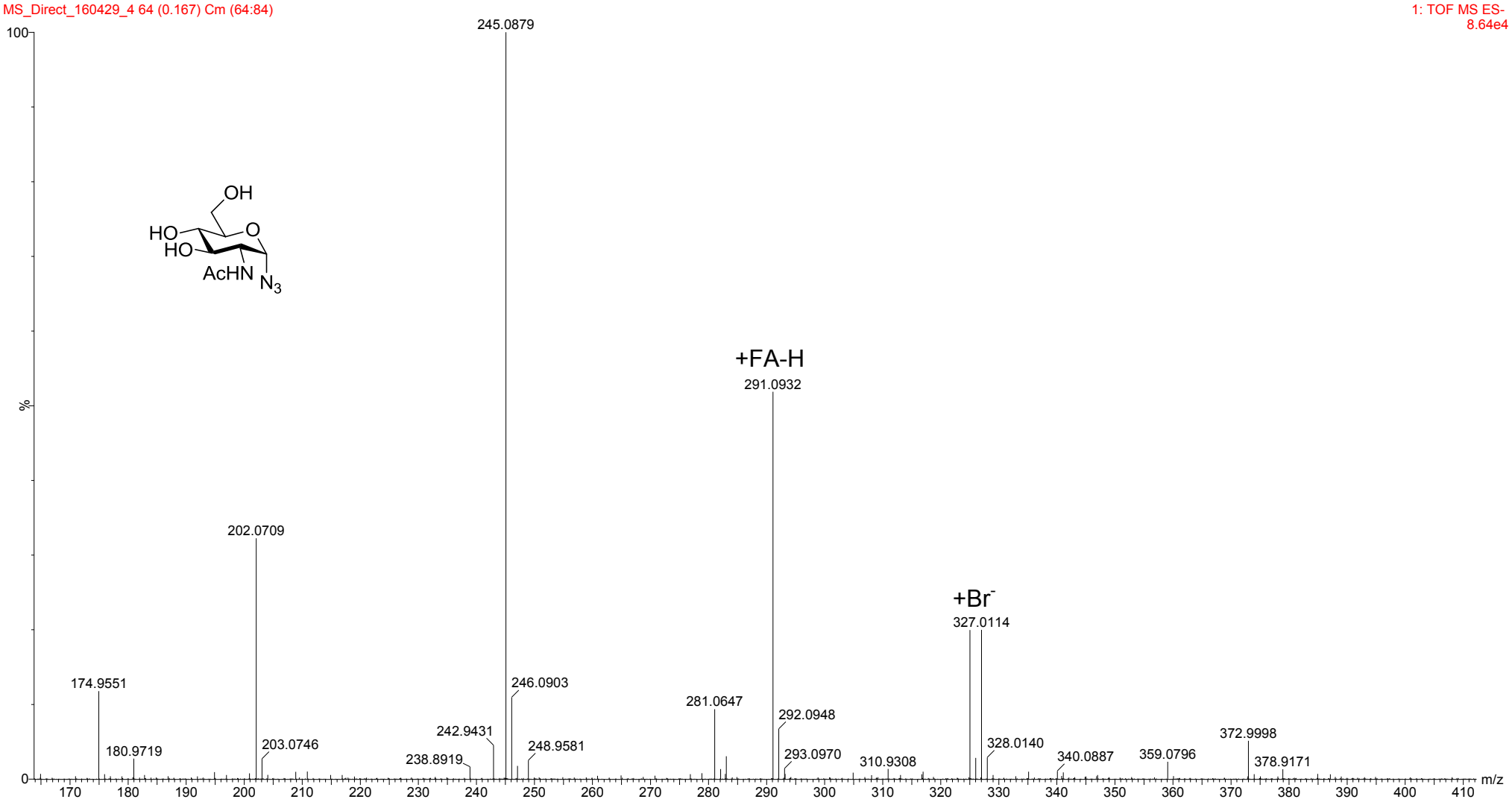
2-Acetamido-2-deoxy-α-D-glucopyranosyl azide: ^{1}H NMR spectrum (300 MHz)



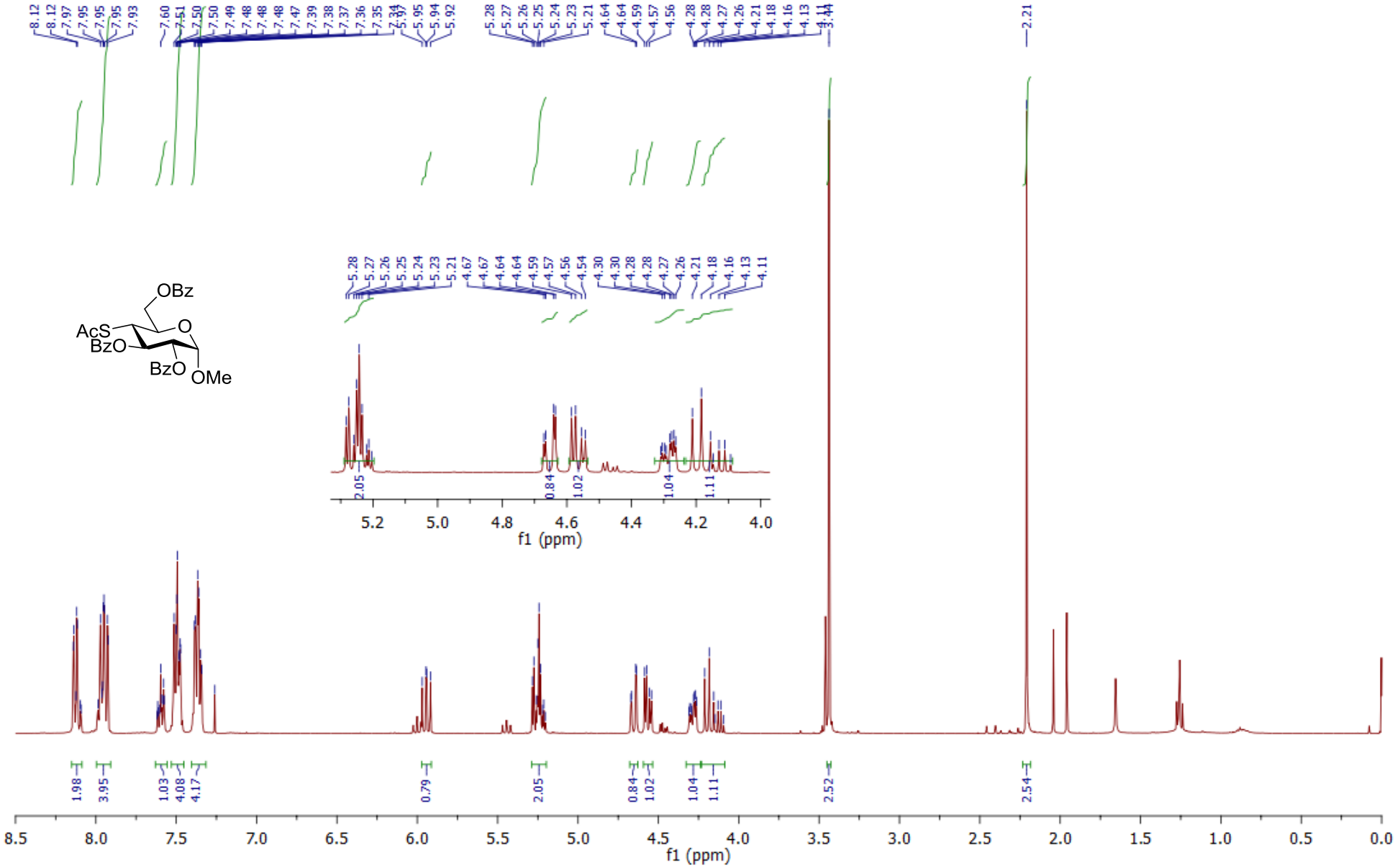
2-Acetamido-2-deoxy-α-D-glucopyranosyl azide: ^{13}C NMR spectrum (75 MHz)



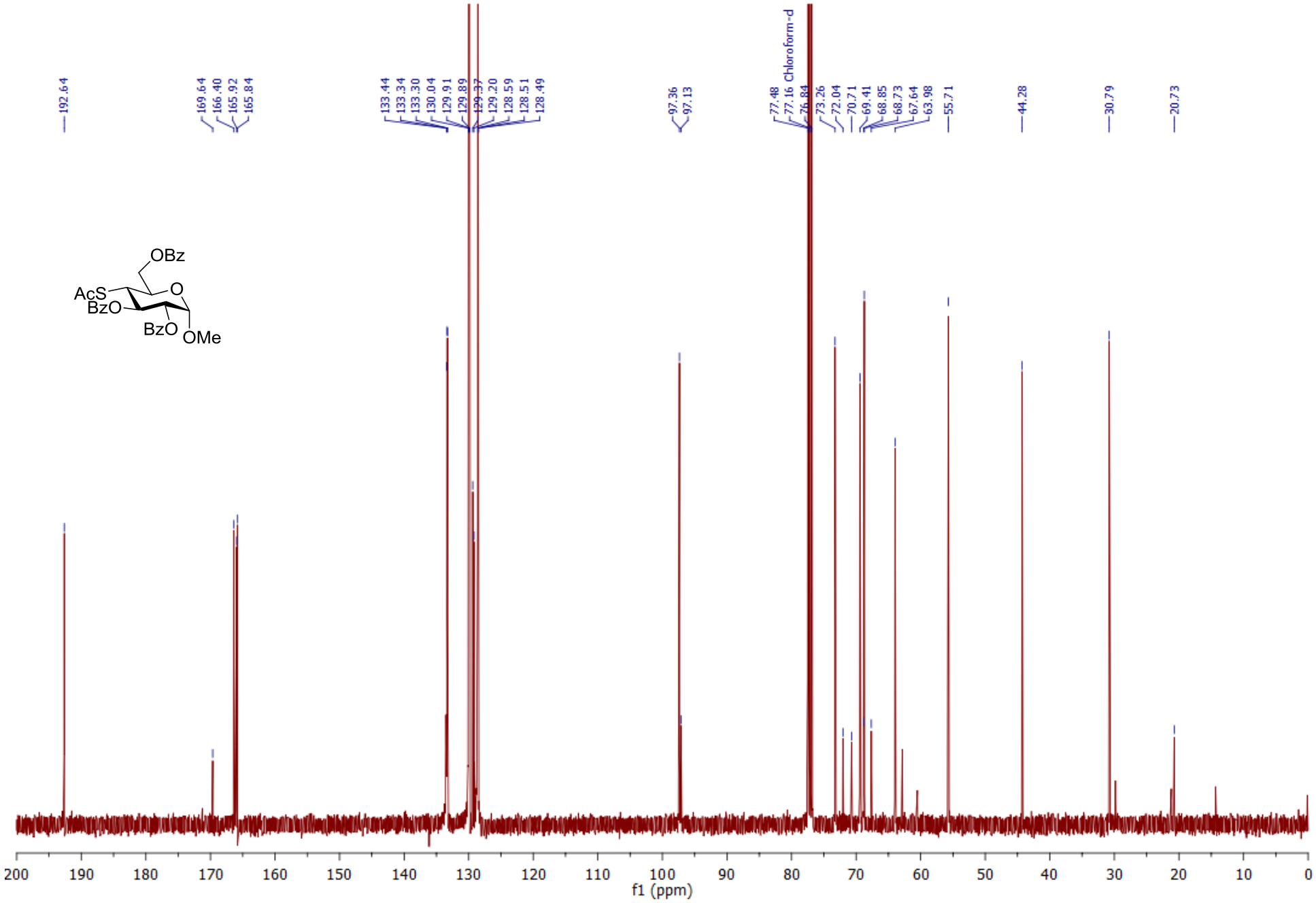
2-Acetamido-2-deoxy-α-D-glucopyranosyl azide: mass spectrum (ESI-TOF-)



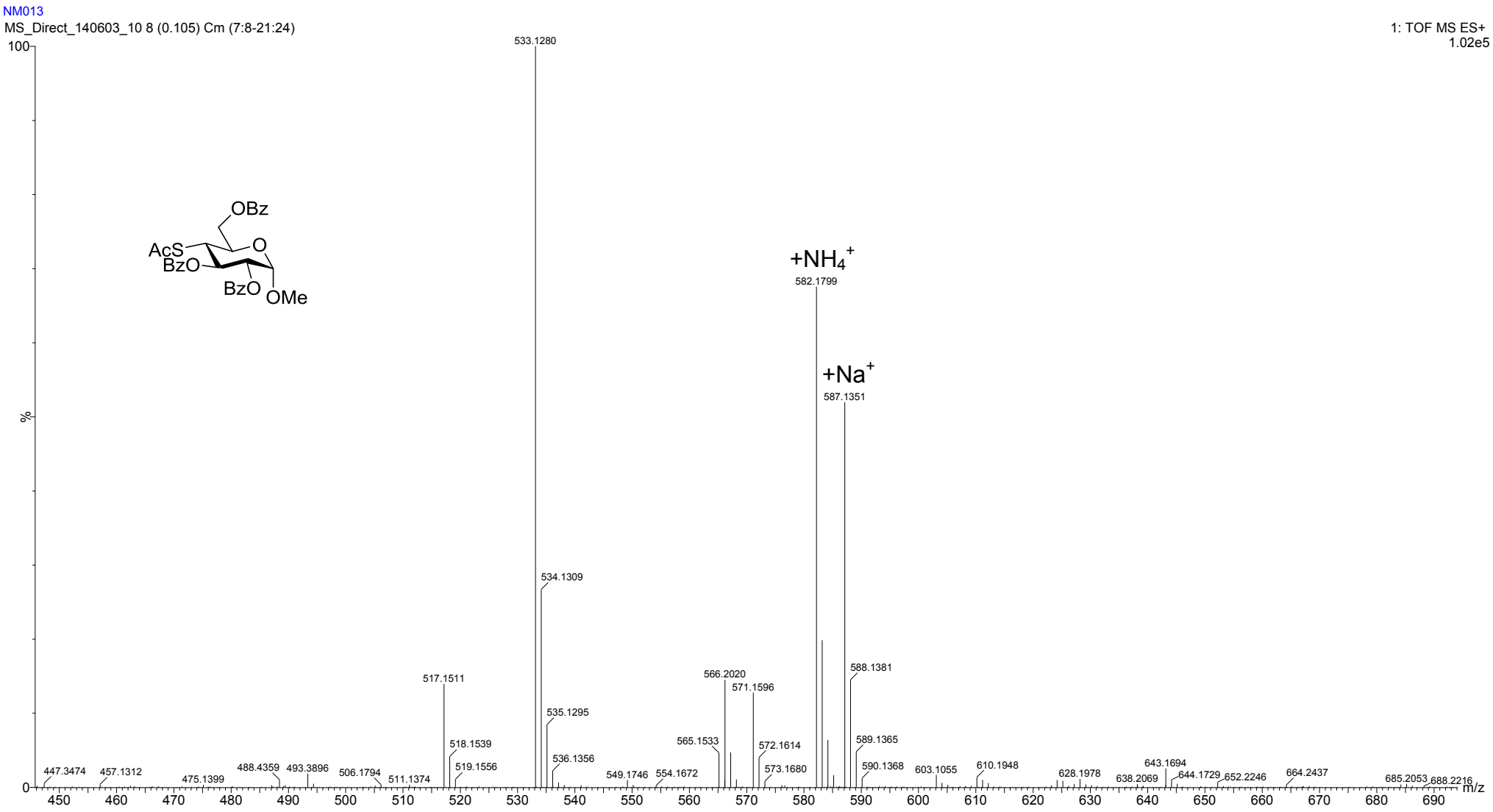
Methyl 4-*S*-acetyl-2,3,6-tri-*O*-benzoyl-4-thio-α-D-glucopyranoside: ^{1}H NMR spectrum (400 MHz)



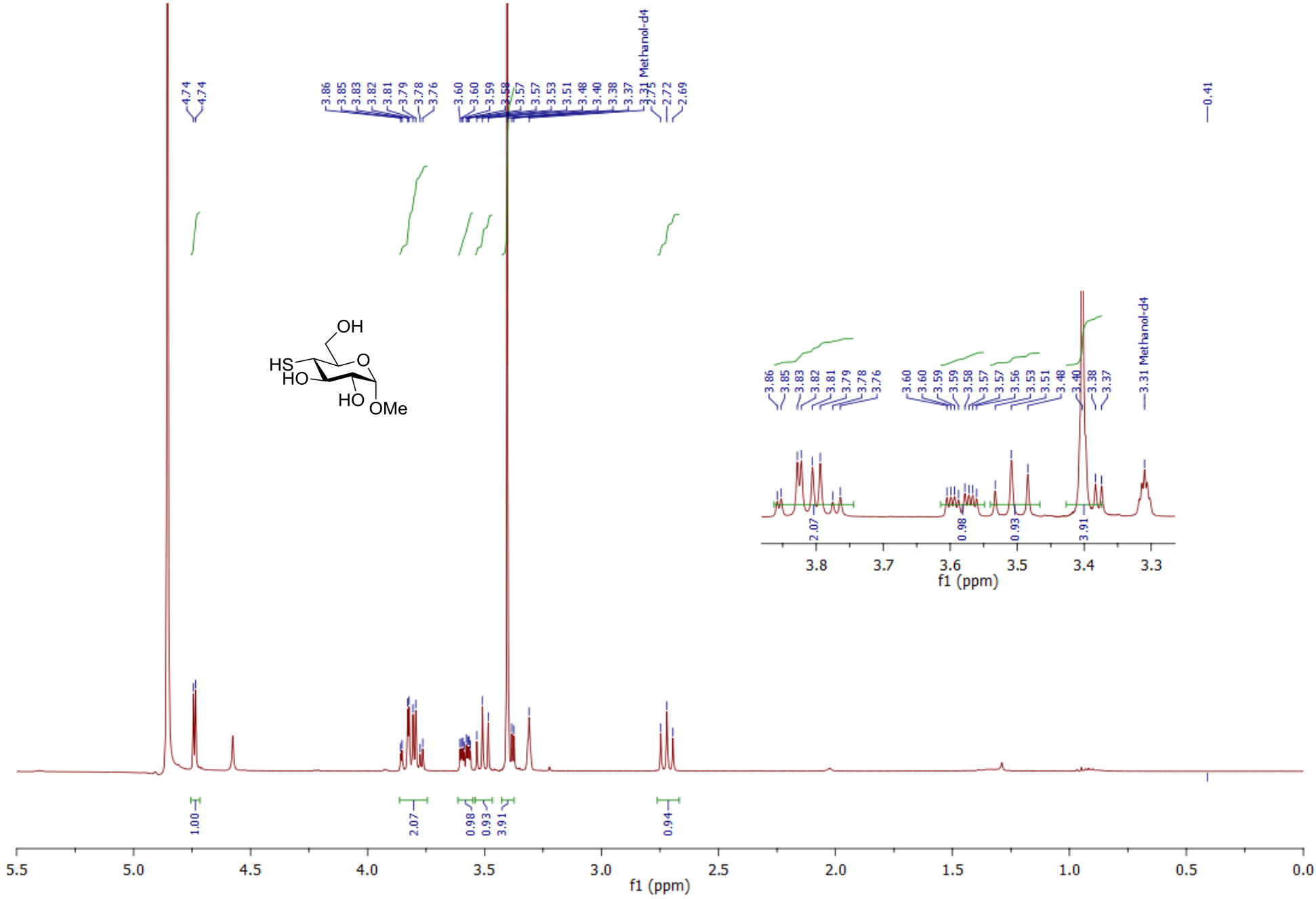
Methyl 4-*S*-acetyl-2,3,6-tri-*O*-benzoyl-4-thio-α-D-glucopyranoside: ^{13}C NMR spectrum (100 MHz)



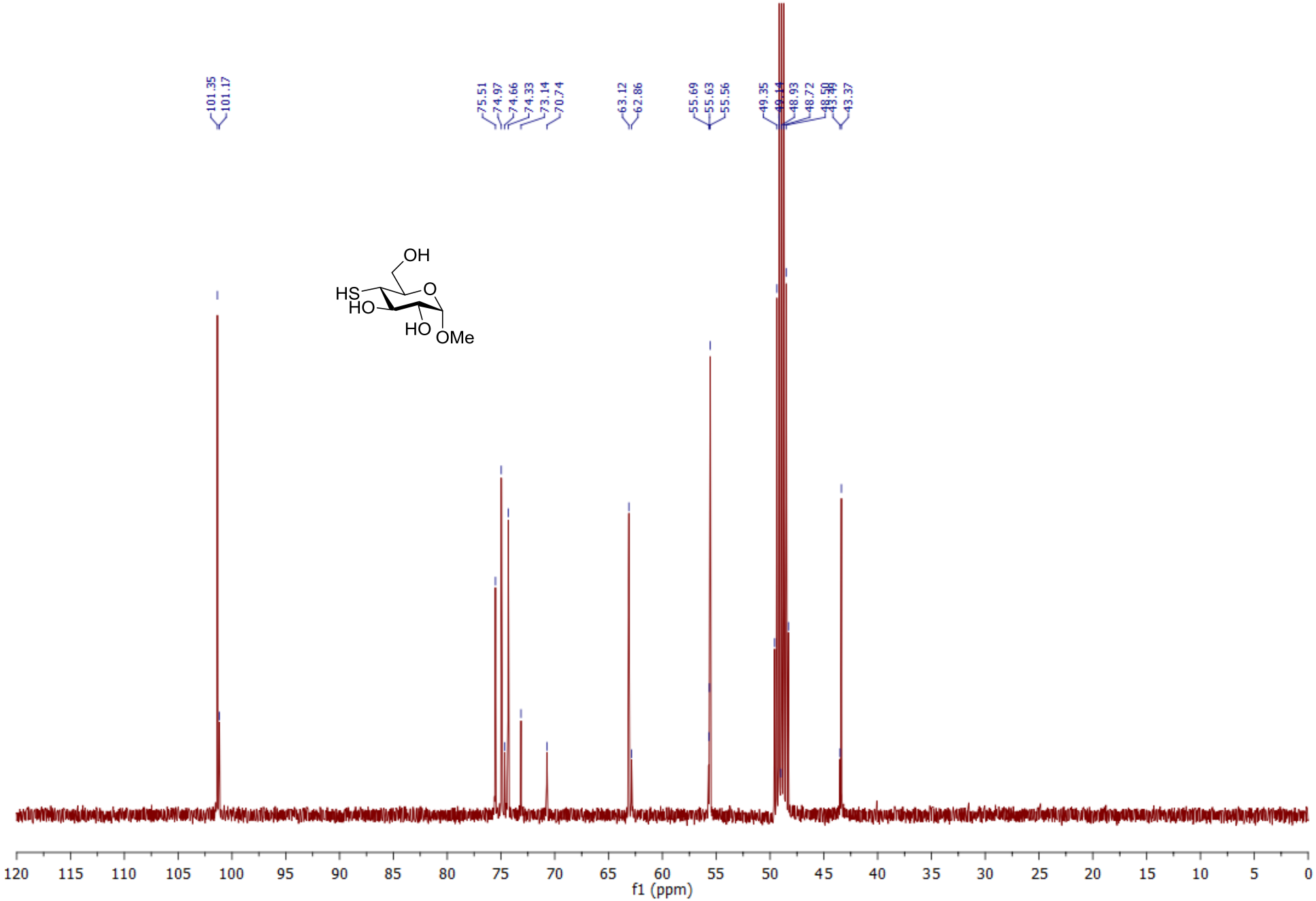
Methyl 4-*S*-acetyl-2,3,6-tri-*O*-benzoyl-4-thio-α-D-glucopyranoside: mass spectrum (ESI-TOF+)



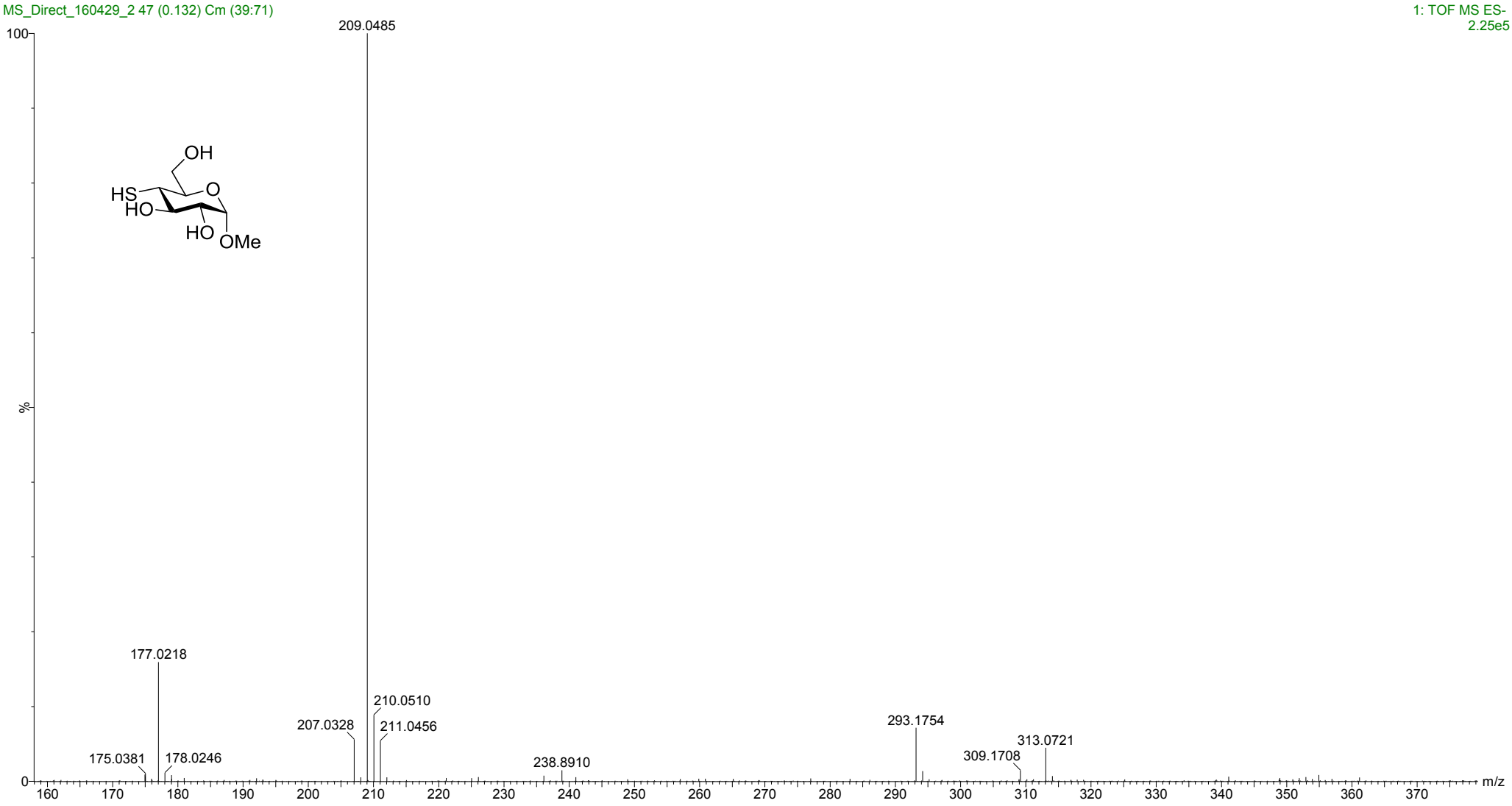
Methyl 4-thio-α-D-glucopyranoside: ^{1}H NMR spectrum (400 MHz)



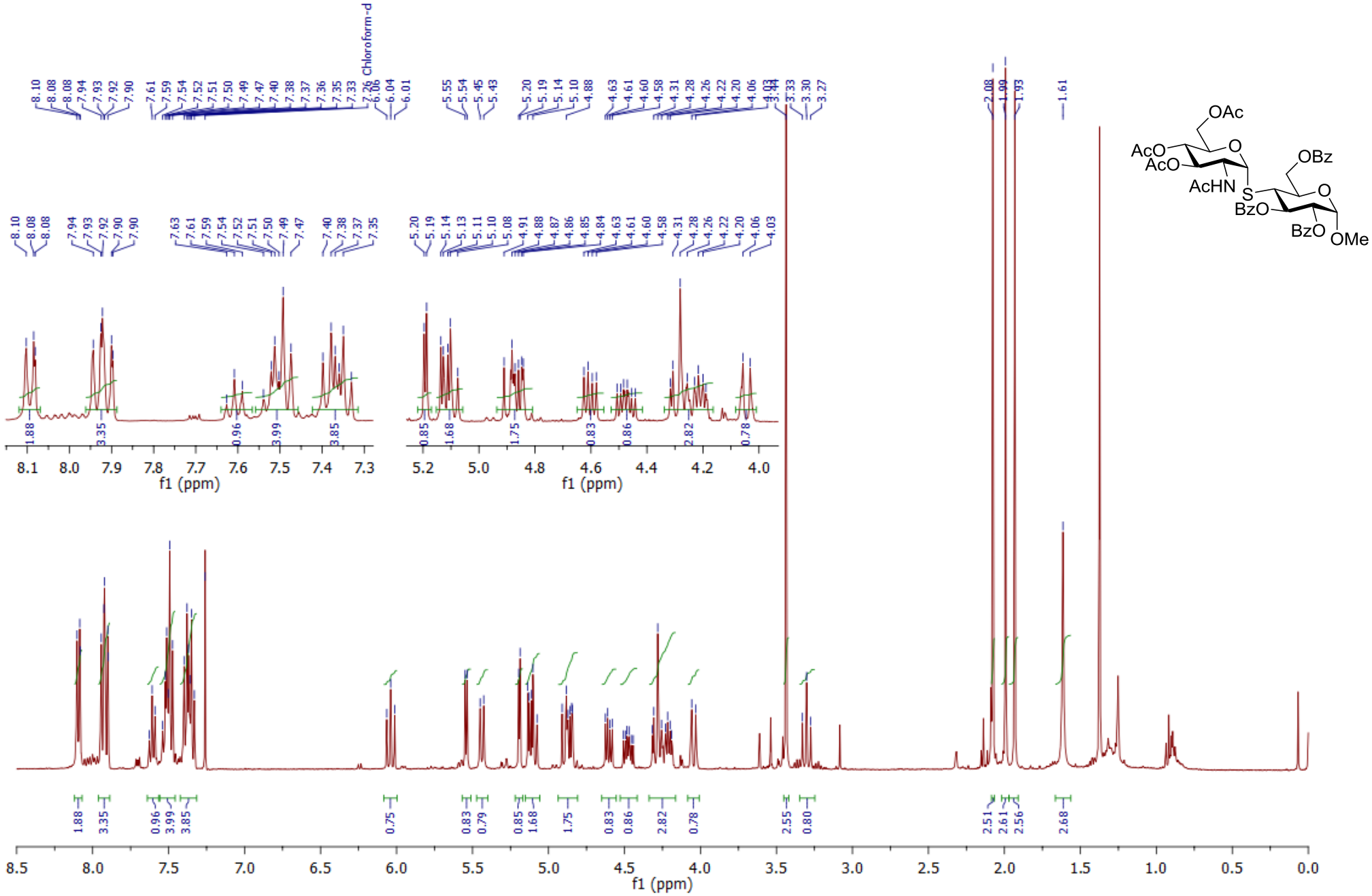
Methyl 4-thio-α-D-glucopyranoside: ^{13}C NMR spectrum (100 MHz)



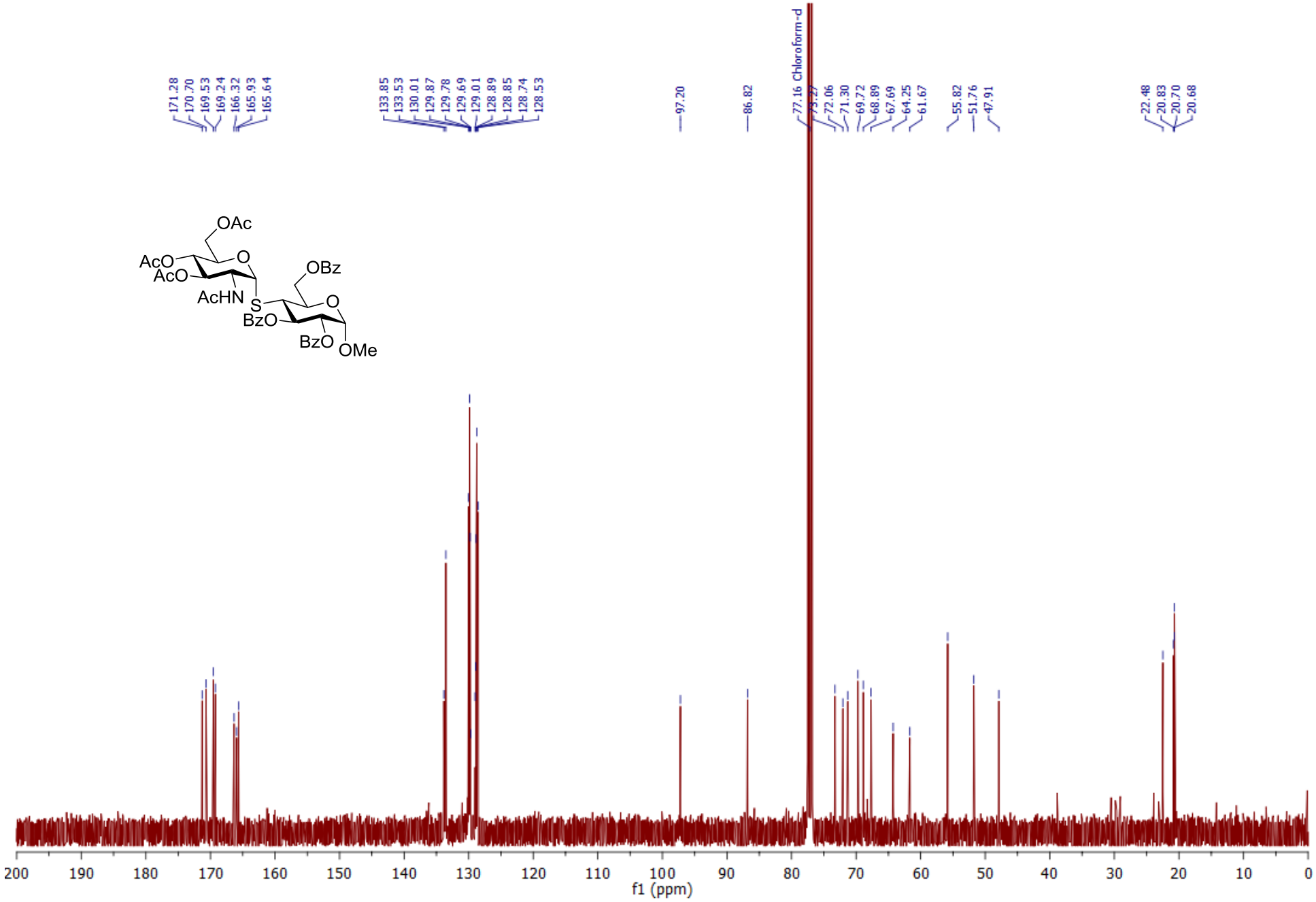
Methyl 4-thio-α-D-glucopyranoside: mass spectrum (ESI-TOF-)



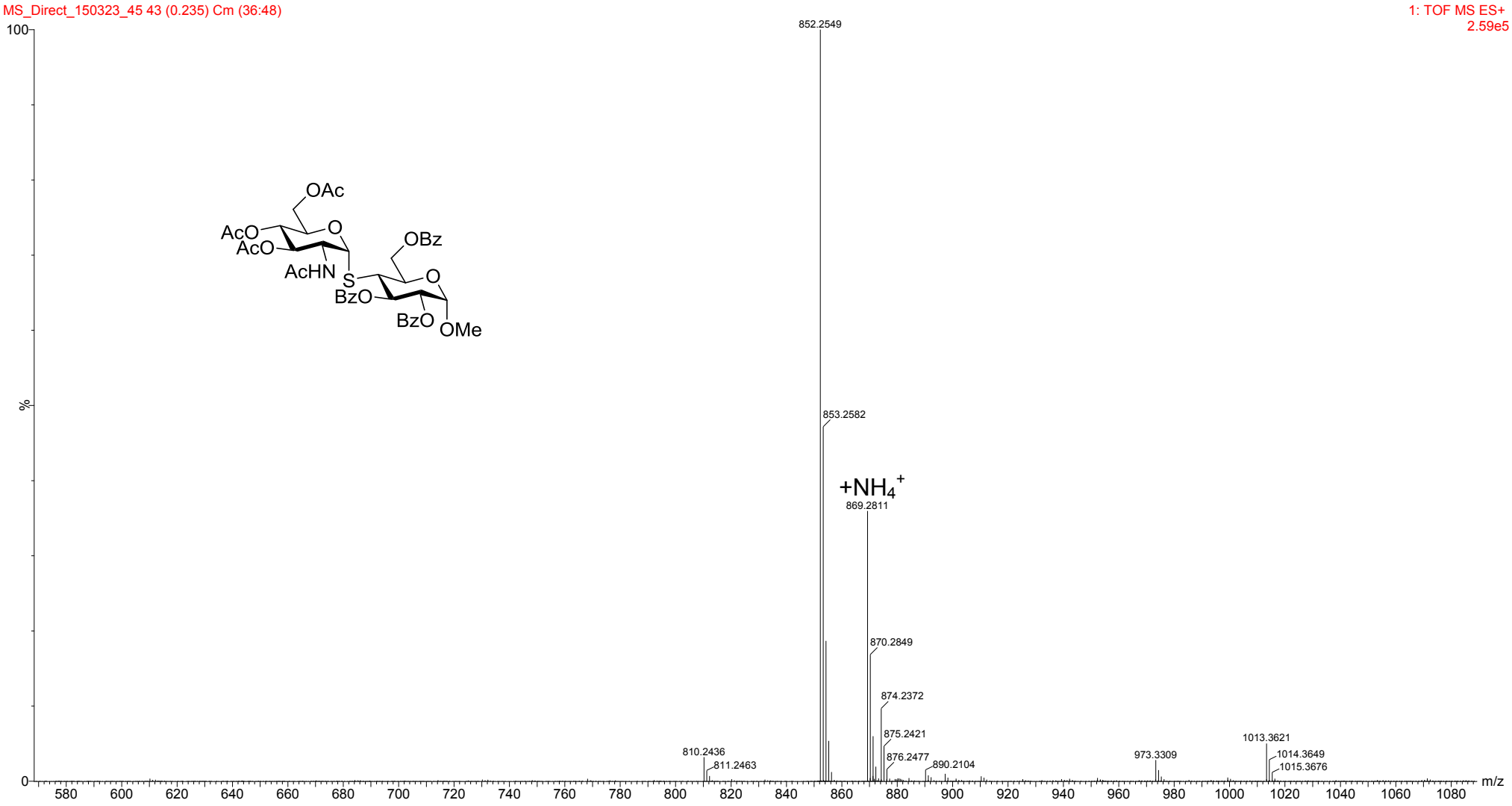
Methyl 4-*S*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-gluco-pyranosyl)-4-thio-3,4,6-tri-*O*-benzoyl-α-D-glucopyranoside: ^{1}H NMR spectrum (400 MHz)



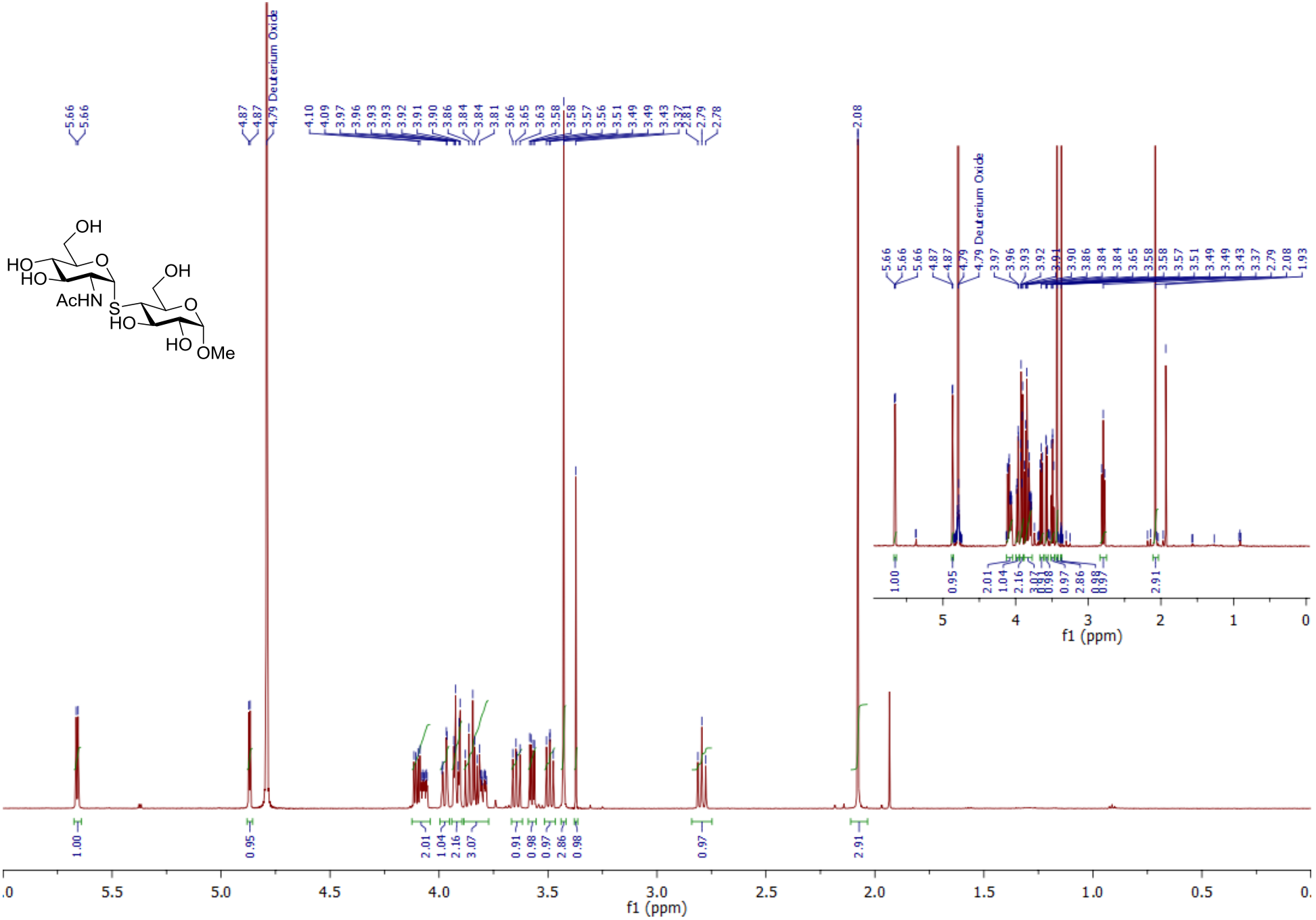
Methyl 4-*S*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-gluco-pyranosyl)-4-thio-3,4,6-tri-*O*-benzoyl-α-D-glucopyranoside: ^{13}C NMR spectrum (100 MHz)



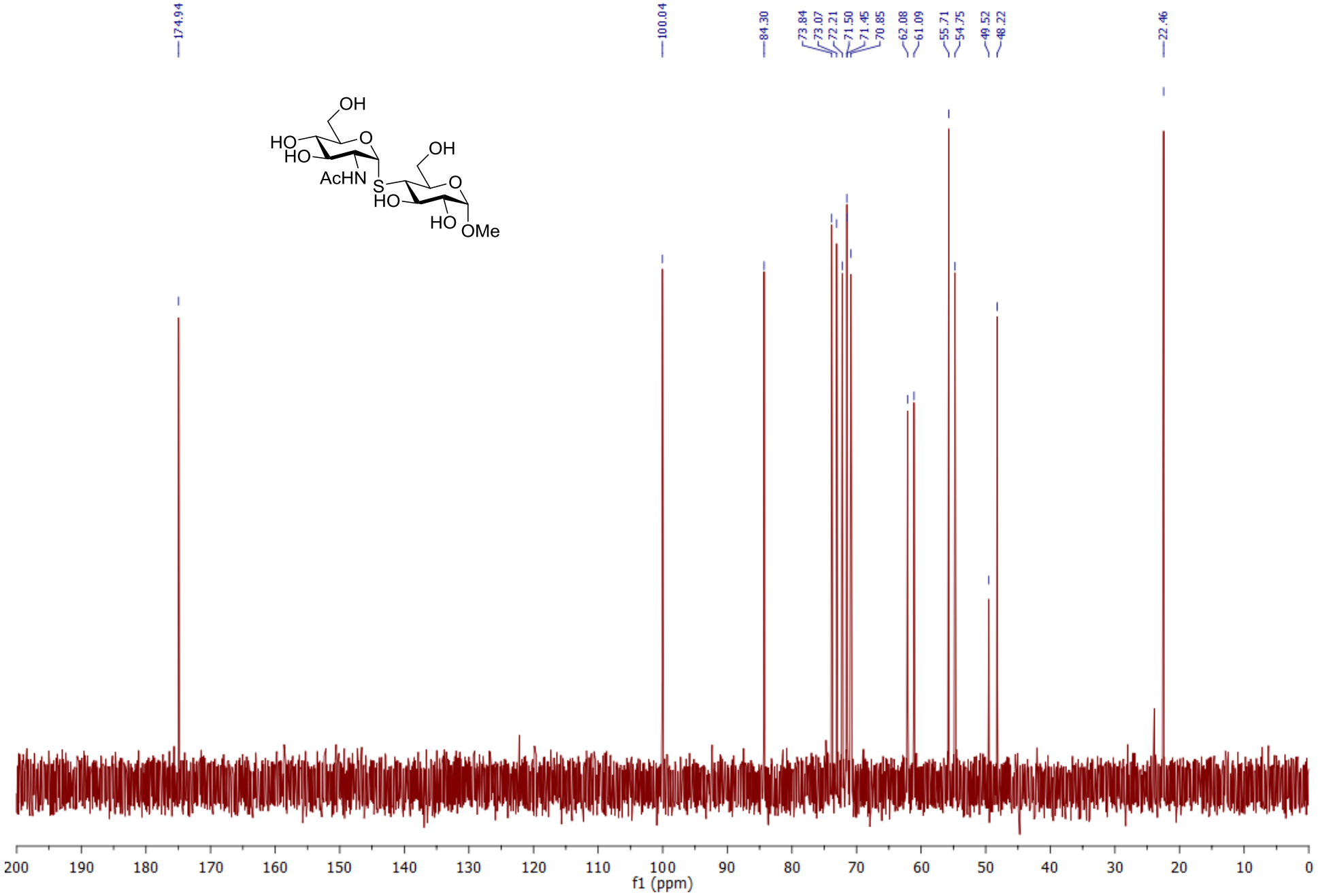
Methyl 4-*S*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-gluco-pyranosyl)-4-thio-3,4,6-tri-*O*-benzoyl-α-D-glucopyranoside: mass spectrum (ESI-TOF+)



Methyl 4-*S*-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-4-thio-α-D-glucopyranoside: ^{1}H NMR spectrum (600 MHz)



Methyl 4-*S*-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-4-thio-α-D-glucopyranoside: ^{13}C NMR spectrum (150 MHz)



Methyl 4-*S*-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-4-thio-α-D-glucopyranoside: mass spectrum (ESI-TOF+)