A Dissertation

Titled:

# Total Synthesis of Zwitterionic Bacterial Polysaccharide (PS A1) Antigen Fragments from B. fragilis ATCC 25285/NCTC 9343 with Alternating Charges on Adjacent Monosaccharides

by

Pradheep Eradi

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Doctor of Philosophy Degree in

Chemistry

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Dr. Peter R. Andreana, PhD, Committee Chair

_____

Dr. Steve Sucheck, PhD, Committee Member

_____

Dr. Jianglong Zhu, PhD, Committee Member

_____

Dr. Amanda C. Bryant-Freidrich, PhD, Committee Member

_____

Dr. Cyndee Gruden, Dean
College of Graduate Studies

The University of Toledo
May 2019

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An Abstract of

Total Synthesis of Zwitterionic Bacterial Polysaccharide (PS A1) Antigen Fragments from B. fragilis ATCC 25285/NCTC 9343 with Alternating Charges on Adjacent Monosaccharides

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Zwitterionic polysaccharides (ZPSs) are a relatively new class of carbohydrate antigens, with a paradigm shifting property; they can activate $CD4^+$ T-cells in the absence of lipids, peptide(s) or protein(s) upon MHC class II presentation. Up until now, various anaerobic bacteria are known to express ZPSs, for example, PS A1, PS A2 and PS B (*Bacteroides fragilis*), Sp1 (*Streptococcus pneumoniae*), CP5 and CP8 (*Staphylococcus aureus*) and *O*-chain antigen (*Morganella morgani*). Among all the afore mentioned ZPSs, Sp1 and PS A1 polysaccharides were the prime focus of research for the past few decades and their biological properties are very well-understood. Polysaccharide A1 (PS A1), the most potent and abundantly expressed in the capsular polysaccharide complex (CPC) of *B. fragilis*, is known for its dual immunomodulatory properties based on the physiological location in the host. PS A1 has been used as a carrier for various tumor antigens like Tn, TF and STn, opening a platform for entirely carbohydrate-based vaccines and the respective immunological outcomes are now known to be very promising. Biological isolation of PS A1 through tedious work-up procedures leads to heterogeneous material in length, and is not suitable for the precise mechanistic studies for T-cell activation and

beyond. A well-thought and executed chemical synthesis can provide completely defined synthetic PS A1 antigen fragments and can be used as molecular probes for the study of T-cell activation and other important biological processes. In order to determine the immunologically active epitope of PS A1, the first total synthesis of PS A1 repeating unit with alternating charges on adjacent sugars was accomplished using a linear glycosylation strategy and biological studies are currently in progress. Synthesis of oligomeric length PS A1 antigen fragments were up-taken to determine whether the immunologically active T-cell epitope of PS A1 is a *sequential* or *conformational* epitope. Pure, well-defined synthetic PS A1 fragments will help in understanding the precise T-cell activation mechanism and provide unprecedented biological data through *in vitro* and *in vivo* studies; access to crystal structures of MHC II proteins with PS A1 synthetic antigen fragment will tremendously help in understanding the nature of critical interactions involved between the peptide and sugar residues. The outcome of the above mentioned studies will lead to the development of completely defined, entirely carbohydrate-based "synthetic vaccine candidate" and also provides opportunity to develop new synthetic PS A1 analogues based on the structure-activity relationship.

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# Table of Contents

# List of Figures

# List of Schemes

# List of Abbreviations

APCs.....Antigen presenting cells
Apa.....Alanine proline rich antigen
AgOTf.....Silver triflate
Abs.....Antibodies

BCRs.....B-cell receptors
BFT.....*B. fragilis* toxin

CPSs.....Capsular polysaccharide complex
CPC.....Capsular polysaccharide complex
CD.....Circular dichroism
cDCs .....Conventional dendritic cells
CAN.....Ceric ammonium nitrate

DCs .....Dendritic cells
DCM .....Dichloromethane

ETBF.....Enterotoxigenic *B. fragilis*
EAE .....Experimental autoimmune encephalomyelitis

GF .....Germ-free
$GHNO_3$.....Guanidinium nitrate

HEL .....Hen egg-white lysozyme

ILs.....Interleukins
iNKT.....Invariant natural killer cells
IBD .....Inflammatory Bowels disease
IFN.....Interferons
ITC.....Isothermal titration calorimetry

LPS .....Lipopolysaccharides

Mt*b*.....Mycobacterium tuberculosis
MHC II.....Major histocompatibility complex
MeOH .....Methanol

NTBF .....Nontoxigenic *B. fragilis*
NBS .....*N*-bromo succinimide

PLA.....Phospholipase A2
PS A1.....Polysaccharide A1
PS A2.....Polysaccharide A2
PS B.....Polysaccharide B
pDCs.....Plasmacytoid dendritic cells

RNS .....Radical nitrogen species

Sp1.....*Streptococcus pneumoniae* 1
STn.....Sialyl Tn
SPR.....Surface plasmon resonance

TD.....T-cell dependent
TI .....T-cell independent
TCR .....T-cell receptor
TLR2.....Toll-like receptor 2
Tn.....Thomsen-nouveau
TF .....Thomsen-Friedenreich
TCA .....Trichloroacetimidate
TMSOTf.....Trimethylsilyl triflate

TEA .....Triethylamine

ZPSs.....Zwitterionic polysaccharides

# List of Symbols

α.....Alpha diastereomeric glycosidic linkage
β.....Beta diastereomeric glycosidic linkage
˚C.....Degrees Celsius
Å.....Angstrom
%.....percentile
©.....Copyright
K.....Kappa
®.....Registered trademark

# Chapter 1

# Zwitterionic Polysaccharides

## 1.1 Introduction – Capsular Polysaccharides

Capsular polysaccharides (CPSs) are expressed on the surface of many known pathogenic bacteria in the form of a capsule, so that they might escape from the process of phagocytosis and be able to survive in the host ecosystem. Tissue fixed macrophages and migrating macrophages are known to clear most of the pathogenic bacteria from the body, as part of the critical defense mechanism exhibited by the host immune system.[1] Pathogenic bacteria CPSs, in general, resist the process of phagocytosis through avoiding complement deposition through several mechanisms such as sub-capsular component masking leading to antibody independent alternative complement pathway, some capsules have sialic acid that to bind the serum proteins[2] and de-amplification of C3B deposition.[1b] Capsular polysaccharides, in general, are high molecular weight polysaccharides consisting of several hundreds of repeating units accounting for the massive molecular weight. The nature of the immune response is dependent on the structural parameters of the capsular polysaccharide, like repeating unit sequence or composition, conformation ($^4C_1$ or $^1C_4$), regio- and stereo-specific nature of the glycosidic linkage. For example,

*Neisseria meningitidis* group B and C have the similar repeating unit monosaccharide composition but differ in the position of hydroxyl groups that form the glycosidic linkage between the sialic acid residues, α(2,8) *vs* α(2,9) linkages respectively. Group C polysaccharide is immunodominant, whereas group B CPS is non-immunogenic.[3] Similarly, group B *streptococcus* type Ia and Ib elicit two distinct immune responses which are not cross protective, reflecting the conserved nature of the immune recognition process due to a minute structural difference in the glycosidic linkage between the sugar residues β(1,4) *vs* β(1,3) respectively.[4] Ovodov *et. al* reviewed the structural features of most bacterial polysaccharides in detail.[5]

In order to protect ourselves from these pathogenic bacteria, our own immune system can be educated to constrain the pathogenic organisms using the principles of vaccines. Until very recent times, carbohydrate antigens were considered poorly immunogenic compared to that of highly immunogenic peptide/proteins.[6] However, in the last few decades, a great deal of work has been focused on understanding the role of carbohydrate antigens on the adaptive arm of the immune system and has led to significant improvement in our understanding of previously under-appreciated class of biomolecules called as glycans.[6-7]

### 1.1.1. Lack of sustained memory: *T-cell independent* nature of glycans:

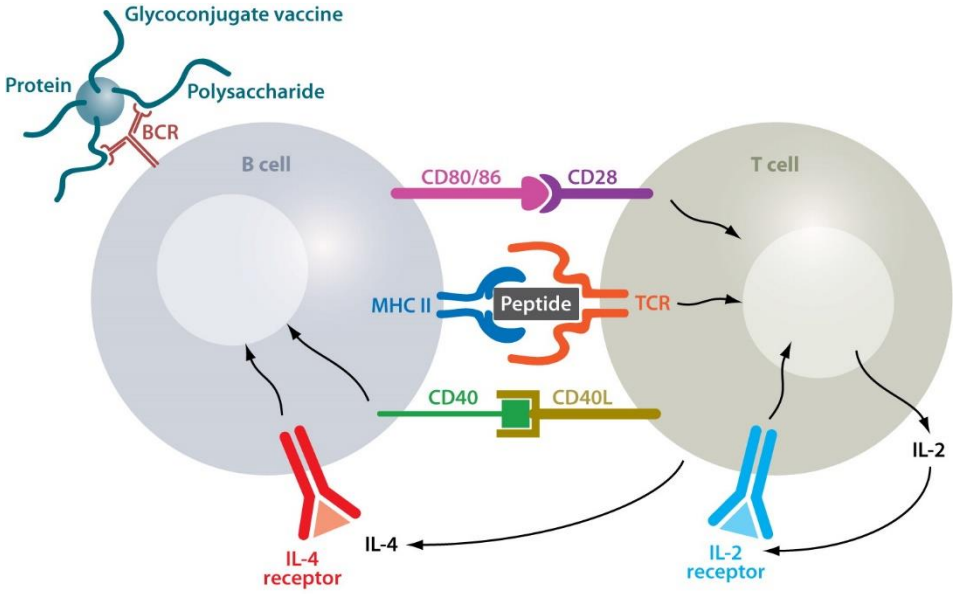
Carbohydrates interact with innate and adaptive arms of the immune system through pathogen associated molecular pattern (PAMP) receptors[8] and B-cell receptors (BCRs) eliciting primarily a weak, T-cell independent immune response lacking sustained memory.[9] Furthermore, antigens interacting with adaptive arm of the immune system are

classified into T-cell dependent (TD) and T-cell independent (TI) antigens, whereas, TI antigens are sub-classified into TI-1 (lipopolysaccharides-LPSs) and TI-2 antigens (capsular polysaccharides-CPSs) respectively.[9] The major impediment for using pure CPSs as vaccines is the lack of sustained T-cell memory, especially in high risk age groups (children below 2 year old and elderly) with no booster immune response and lack of isotype switching (IgM to IgG). The pioneering work of Avery and Goebel[10] in the field of glycoimmunology showcased that the antigenicity of glycan components can be enhanced by the conjugation of glycans to proteins, which will lead to the generation of highly glycan-specific antibodies (Abs).

### 1.1.2. Glycoconjugate vaccines – *T-cell dependent* response for glycans:

Using hapten-carrier conjugation principle,[11] CPSs of various bacteria have been conjugated to highly immunogenic proteins and promising results have been obtained.[12] Pure CPSs induce B-cell mediated, highly diverse and low affinity IgM antibody production, whereas CPSs conjugated to protein (glycoconjugate) can elicit T-cell help leading to IgM to IgG class switching, production of interleukins (ILs), cytokines and result in a sustained memory responses. As per the current understanding of glycoconjugate based T-cell activation mechanism,[6, 13] the glycoconjugates are uptaken by the antigen presenting cells (APCs) into the endosomes, fragmented into smaller fragments and presented in the context of major histocompatibility complex class II protein (MHC II) at the cell surface of APCs (Figure 1). The glycopeptide antigen presented by the MHC II complex will be recognized by the αβ T-cell receptor (TCR) of $CD4^+$ T-cells along with co-stimulation (CD80/86 and CD28) leading to the secretion of IL-2 and upregulation of

IL-2 receptor resulting in the activation of the T-cell. Activation of T-cell results in the production of IL-4, upregulation of CD40 ligand, IL-4 receptor on the B-cells binds to IL-4 and leads to the upregulation of CD40. The interaction of CD40-CD40L in combination with IL-4 signaling leads to the activation of B-cell (Figure 1). Activation of B-cell mediated through T-cell results in IgM to IgG class switching, leading to the production of high affinity, glycan specific IgG antibodies, memory B-cells and plasma cells. The above hypothesis was put into practical test and the precise mechanistic details involved in the glycoconjugate based activation of T-cell are not still clear enough.[13-14]




Avci FY, Kasper DL. 2010.
Annu. Rev. Immunol. 28:107–30

**Figure 1 - 1*:** Mechanism of $CD4^+$ T-cell activation by glycoconjugate vaccine.

*"Reproducedwith permission from Avci, F. Y.; Kasper, D. L. *Annu. Rev. Immunol.* **2010**, *28*, 107. Copyright© 2010 Annual reviews"

Recently, Kasper *et. al* showcased the importance of the polysaccharide structure on the T-carb response, where group C polysaccharide of *N. meningitidis* (MenC) failed to elicit T-carb response due to the instability of polysaccharide to acid hydrolysis in endosomes, in contrast to other glycoconjugates tested (Vi, GBSIb ad Hib) which resulted in specific T-carb response.[15]

### 1.1.3. Glycoantigen interaction with adaptive immune system:

Avci *et. al* recently reviewed various types of carbohydrate antigen interactions in a T-cell dependent manner,[7] highlighting various new studies involving glycopeptides, glycolipids, glycoconjugates and zwitterionic polysaccharides (ZPSs). In the precedented literature, many examples have highlighted the importance of glycans in a carbohydrate specific T-cell response. Using natural or synthetic glycopeptides, the influence of glycan structure and the site of glycosylation on different glycopeptide/proteins has been studied. Deck *et. al* showed that the site of covalent linkage (Ser residue) and amino acid residue specificity (Ser56) of glycan galactobiose (a disaccharide) on a MHCII binding protein HEL (hen egg-white lysozyme) was critical for T-cell hybridoma recognition.[16] Studies have shown the effect of degrees of glycosylation and length of glycan on a glycopeptide towards the nature of T-cell effective response.[17] Glycoproteins like type II collagen,[18] bee venom allergen phospholipase A2 (PLA),[19] alanine proline rich antigen (Apa) from *Mycobacterium tuberculosis* (*Mtb*),[20] lipoglycoprotein[21] (LprG) from *Mycobacterium leprae* and *Mtb* are known to induce glycan specific T-cell ($CD4^+$ or/and $CD8^+$) response.

Glycolipids, for example α-galactosylceramide (α-GalCer), are known to elicit a specific immune response through the activation of invariant natural-killer T-cells (iNKT)

when presented in the context of CD1d molecule (MHCI type protein).[22] The crystal structure of CD1d molecule with glycosylceramides have illustrated that the lipid fitting into the binding pocket of CD1d molecule exposes the glycan portion to the TCR of natural killer cells[23] with higher affinity and longer half-lives compared to $CD4^+$ and $CD8^+$ T-cell receptors binding to peptide antigens presented through MHC II and MHC I molecules respectively.[24] The outcome of iNKT cells activation by glucosylceramides leads to direct cytotoxicity, targeting and killing cancer cells.[25] α-GalCers are used as adjuvants because of their ability to enhance the antigenicity of various target entities.[26] Capsular polysaccharides of *S. pneumoniae* conjugated to alpha galactosylceramide (αGalCer) had been shown to elicit carbohydrate specific, potent immune response against *S. pneumoniae* infection in mice through the production of high affinity IgG antibodies and memory B-cells.[27]

Glycoconjugate vaccines are very well-known to elicit T-cell-based adaptive immune responses specific to the glycans conjugated to various carrier proteins. Previously, it was thought that only peptide fragments are capable of binding in the MHC I or MHC II binding grooves and carbohydrates do not. In 2011, Avci *et. al* unraveled the previous hypothesis and proposed a new working model for the activation of T-cells by glycoconjugate vaccines.[13] Even though pure polysaccharide vaccines against some of the serotypes of *S. pneumoniae, S. typhi* and *N. meningitidis* being currently used have shown success in adults, they have failed to induce protective responses in infants, elderly and immunocompromised patients.[28] Glycoconjugate vaccines against *S. pneumoniae, N. meningitidis* and *H. influenzae* have been highly successful with regard to their to ability to activate the adaptive arm of the immune system through eliciting T-cell help for B-cells,

producing long lasting memory B-cells, plasma cells, high affinity IgG antibodies through class switching and hence are successful to protect infants and elderly compared to the pure CPSs based vaccines in the market.[28-29]

A new class of molecules called as zwitterionic polysaccharides (ZPSs) are known to elicit a T-cell based immune response solely in the absence of any type of carrier protein or peptide epitope. To date, certain bacterial strains are known to produce zwitterionic polysaccharides; *S. pneumoniae* type I (Sp1),[30] type 5 (CP5) and type 8 (CP8) capsular polysaccharides from *Staphylococcus aureus*,[31] *O*-chain antigen from *Morganella morgani*,[32] polysaccharide A1 (PS A1) from *Bacteroides fragilis* ATCC 25285/NCTC 9343,[33] polysaccharide A2 (PS A2) from *Bacteroides fragilis* 638R[34] and polysaccharide B (PS B) from *B. fragilis* NCTC 9343,[6] among which polysaccharides PS A1 and Sp1 are the most well studied ZPSs (Figure 2).[33] In general, the majority of capsular polysaccharides found on bacterial surface are basically neutral or negatively charged and studies on these neutral/negatively charged CPSs have shown their T-cell independent nature.[5-6] In contrast to non-zwitterionic polysaccharides, zwitterionic polysaccharides (ZPSs) are processed through a novel iNOS mediated radical nitrogen species (RNS, specifically nitric oxide $NO^{\cdot}$) in the endosomes, presented in the context of MHC II and recognized by αβ TCR of $CD4^{+}$ T-cells and elicit a robust response with the production of high affinity IgG antibodies through class switching and long-lasting memory B-cells specific for the carbohydrate or glycan portion.[6, 35]

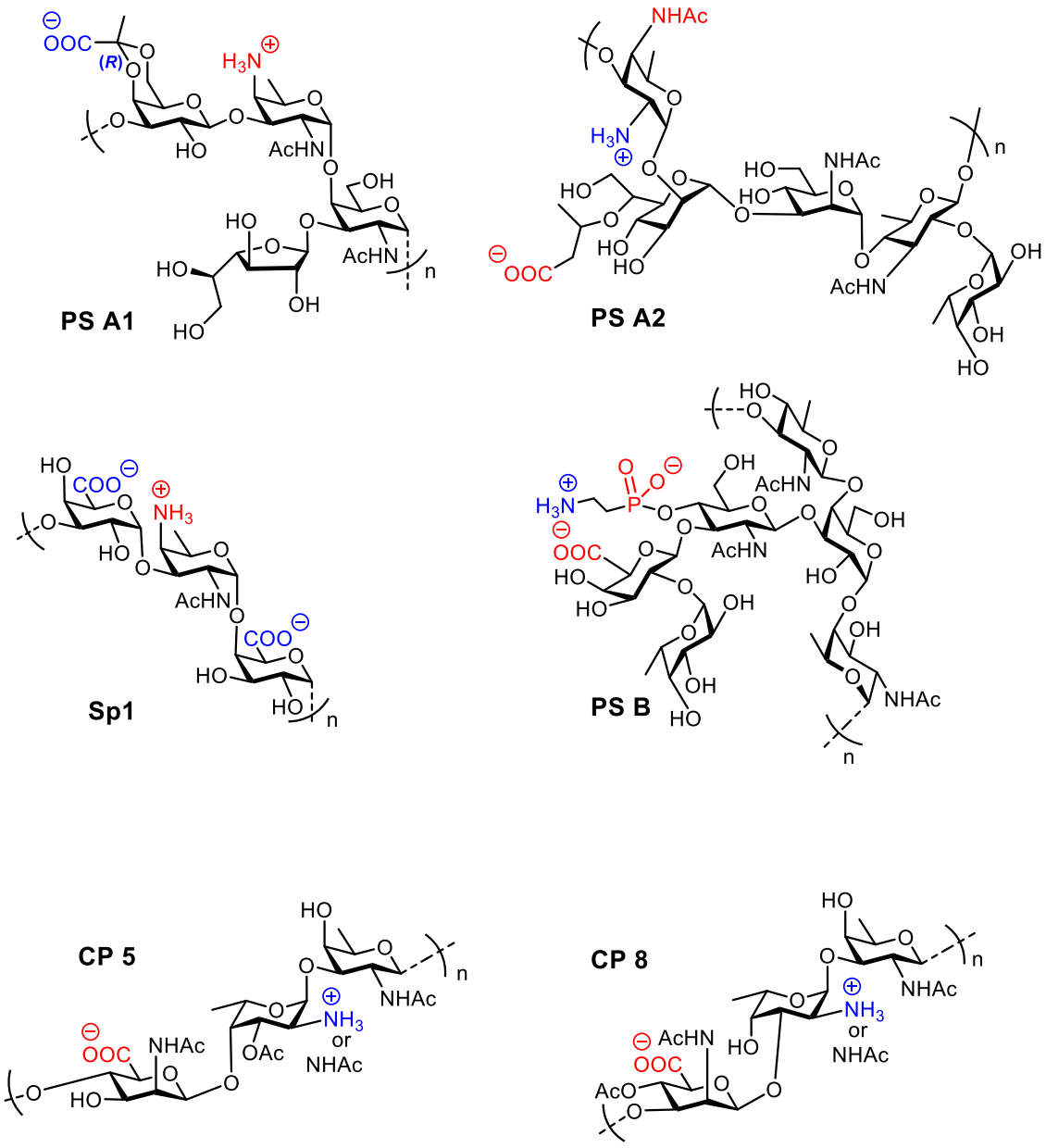



**Figure 1 - 2:** Zwitterionic polysaccharides (ZPSs).

*"Adapted with permission from Eradi, P.; Ghosh, S.; Andreana, P.R. *Org. lett.* **2018**, *20*, 4526-4530. Copyright© (2018) American Chemical Society"

Overall, summarizing the various glycoantigens (glycoprotein, glycolipid, glycoconjugates and zwitterionic polysaccharides) interaction with adaptive arm of the

immune system in a T-cell specific manner (Figure 3) has showcased the importance of understanding the exact role of these previously less appreciated biomolecules called "glycans".

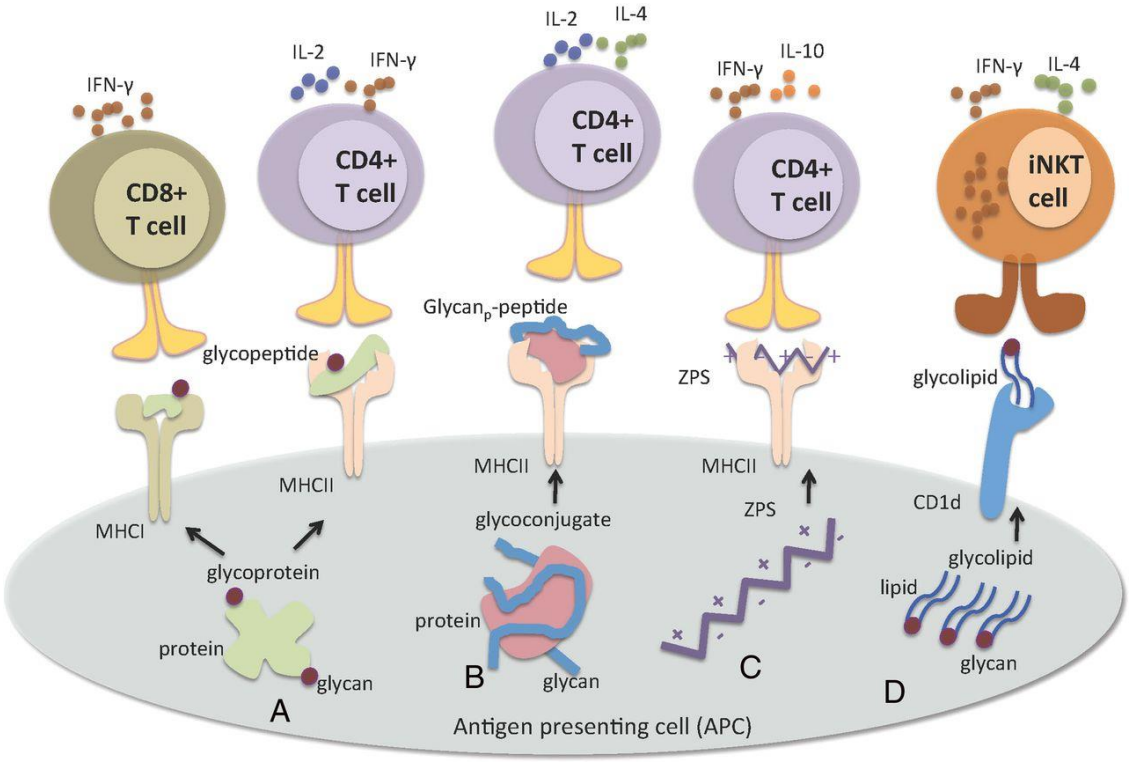



**Figure 1 - 3*:** Various glycoantigen(s) mediated T-cell activation.

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Understanding the precise and specific mechanistic details of T-cell activation by glycans, will reflect a new paradigm in developing rational vaccine constructs with high efficacy rather than irrational use of vaccine constructs (structural changes) based on the immunological outcomes.

## 1.2. *Bacteroides fragilis*: Polysaccharide A1 (PS A1)

### 1.2.1. Introduction:

In 1876, the pathogenic role of the microorganisms was first demonstrated by Robert Koch.[36] Up until then, most of the focus was on the study of pathogenic nature of microbiota with the host.[37] Until recently, focus has been slowly shifting towards the understanding of the symbiotic relation between microbiota and host. Based on the studies related to symbiotic relationship between the host and the bacteria, it has become clear that microbiota influence the hosts well-being in many different ways and play critical roles in the immune system development and hence the concept of "hygiene hypothesis" was proposed due to the significant impact of the microbiota on the well-being of the host/humans.[38] It is a very well-known fact that the number of eukaryotic cells of the host are outcompeted by the prokaryotic cells;[39] microbiota have several ecological niches in the human body like gastrointestinal, respiratory, urogenital tract, oral cavity and on the skin surface. Among all the afore mentioned physiological sites, the major portion of microbiota resides in the gut, known to assist the host in digestion, development of immune system and also on outcompeting the space for harmful pathogenic bacteria.[40] Disruption of the gastrointestinal microbial ecosystem leads to several diseases like inflammatory bowels disease (IBD),[41] colon cancer, asthma,[42] multiple sclerosis,[43] Alzheimer's disease[44] and other metabolic disorders.[45] A large amount of epidemiologic data has shown an inverse correlation between the occurrence of autoimmune or other diseases and very early exposure to microorganisms.[38a-c] As mentioned before, the gastrointestinal tract has more microbes than other and bacteria outnumber other microbes like bacteriophages, protozoa,

viruses and fungi.[46] The gastrointestinal portion is known to host nearly 2172 bacterial species belonging to five phyla, namely, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria and Firmicutes.[47]

### 1.2.2. *B. fragilis* - A commensal gut bacteria:

*Bacteroides fragilis* is a gram-negative, obligate anaerobic gut commensal bacteria known to have dual immunomodulatory properties (pro-inflammatory and immunoregulatory) dependent upon the site of location of *B. fragilis* i.e., inside or outside of the gut.[48] *B. fragilis* comprises only 1-2% of the gut microbiota, and surprisingly being the most common isolated bacteria from intra-abdominal abscess, reflecting its role in the formation of abscess.[48a] Intraperitoneal (IP) treatment of mice with *B. fragilis* and sterile cecal contents elicited the formation of abscess, importantly, the capsular polysaccharide complex (CPC) is known to be critical in the abscess induction.[49] Intraperitoneal implantation of *E.coli* capsular polysaccharides or *S. pneumoniae* polysaccharides (heat killed), failed to induce intraabdominal abscess, showcasing the specificity towards *B. fragilis* CPC.[49a] Pretreatment of mice with pure CPC of *B. fragilis* without the adjuvant (sterile cecal contents) lead to a protective response from abscess formation when challenged with *B. fragilis* or other encapsulated bacterial species, reflecting the importance of *B. fragilis* CPC as a vaccine candidate against intraperitoneal (IP) infectious diseases.[50] Based on the rat model studies to figure out the protective property of *B. fragilis* CPS towards abscess formation, it was clear that the observed protection from the formation of abscess is mediated through adaptive or cellular immunity rather than humoral immunity (transfer of antibodies *vs* splenocytes to naïve mice from immunized

individuals).[51] Later, specific transfer of αβ TCR $CD4^+$ T-cells conferred protection through secretion of anti-inflammatory cytokine IL-10 by the activation of $Foxp3^+$ regulatory T-cells.[52]

Symbiotic interactions of *B. fragilis* with the host is largely dependent on the highly dynamic and complex structure of the capsular polysaccharide complex (CPC).[53] *B. fragilis*, capable of producing eight different capsular polysaccharides, namely polysaccharide A (PS A) to polysaccharide H (PS H), a rare phenomenon observed in various bacterial species and among the eight different polysaccharides expressed, PS A1 is the most abundant and bioactive polysaccharide. *B. fragilis* can produce any of the afore mentioned eight different polysaccharides (PS A-H) to maintain the dynamic nature of the surface polysaccharide complex through a process of multiple DNA inversions.[54] A major fraction of the *B. fragilis* genome is dedicated to the polysaccharide production, glycan metabolism and is known to be very important for the survival of bacteria by evoking complement mediated phagocytosis,[55] to successfully interact with the host immune system.[56] Recently Neff *et. al* attempted genomic screen strategy and identified various bacterial species capable of producing zwitterionic polysaccharides (ZPSs) apart from the well-known ZPSs producers *B. fragilis, S. pneumoniae* and showcased the induction of higher levels of T-regulatory cells (T-regs), anti-inflammatory cytokine IL-10 resulting in protection against experimental colitis in mice models.[57] The zwitterionic property observed in PS A1, Sp1 and PS A2 polysaccharides is due to the presence of the rare amino sugar D-AAT, synthesized by the gene *wcfR* and is transferred to the backbone of the polysaccharide by the gene adjacent to the wcfR gene, named *wcfS* resulting in the presence of D-AAT at variable positions in the above mentioned structurally different ZPSs.[57] The

dual immunomodulatory properties of *B. fragilis* is attributed to the abundantly expressed and most potent polysaccharide A1 (PS A1).[6]

### 1.3. Polysaccharide A1 (PS A1): Immunomodulatory factor of *B. fragilis*

Among the eight different polysaccharides (PS A – PS H) expressed by *B. fragilis*, polysaccharide A1 is the most abundantly expressed and highly potent (pure PS A1) in comparison with the whole capsular polysaccharide complex (CPC).[58] Mutant species, not capable of producing the eight different polysaccharides or capable of producing only few of the eight polysaccharides are not able to colonize successfully and thus reflecting the importance of structural diversity of CPC.[59] PS A1 polysaccharide is known to induce the formation of abscess through T-cell activation, can bind to Toll-like receptor 2 (TLR-2) on dendritic cells (DCs)/T-cells and leads to the differentiation of $CD4^+$ T-cells into T-helper 1 cells (Th1) secreting IFN-γ, resulting in innate and adaptive arm coordination.[60] PS A1 is known to play an direct role in the process of lymphoid organogenesis, systemic T-cell deficiency correction and immune system maturation in a proper way.[61] PS A1 can activate a subset of T-regs ($Foxp3^+$), secreting the anti-inflammatory cytokine IL-10 and hence can lead to the down regulation of various diseases like inflammatory bowels disease (IBD),[52b] asthma[62] and autoimmune encephalomyelitis.[63] All the above immunomodulatory properties can be attributed to the unique structure of PS A1, with alternating charge character on each repeating unit, and leading to a three-dimensional, α-helical type structure with charges exposed on the surface for interaction with αβ TCR of $CD4^+$ T-cells.

#### 1.3.1. Structural features of PS A1:

The unique bio-properties of polysaccharide A1 (PS A1) can be attributed to the three-dimensional structure of the polysaccharide with each repeating unit containing alternating/zwitterionic charge character in contrast to the most bacterial polysaccharides containing either neutral or negative charge.[6] NMR studies lead to the elucidation of the polysaccharide structure with more than 100 repeating units, with nearly 110 kDa molecular weight on average, with each repeating having an unique chemical composition (→3)-α-D-AAT*p*-(1→4) [β-D-Gal*f*(1→3)]-α-D-Gal*p*NAc(1→3)-β-D-Gal*p*(1→) consisting of a tetrasaccharide with positive charge on rare amino sugar D-AAT*p* and negative charge on pyruvate D-Gal*p*.[64] The zwitterionic character is mandatory for PS A1 to be functional in the context of MHC II mediated T-cell activation, *N*-acetylation of the amino group on D-AAT*p* or reduction of the carboxylic functionality on pyruvate D-Gal*p* by carbodiimide reduction leads to complete loss of the T-cell activating property due to the inability of PS A1 to bind and be presented through MHC II complex.[65] Confocal microscopy studies have shown that the zwitterionic charge is not necessary for uptake of PS A1 into the endosomes by APCs, but is mandatory for MHC class II presentation.[66] General mechanism of PS A1 presentation involves, uptake and processing of polysaccharide into smaller fragments of ~15 kDa by radical nitrogen species in endosomes and then these fragments bind to the HLA-DR class II proteins and presented to αβ TCR of $CD4^+$ T-cells.[35a] Even though several studies have been done on the understanding of ZPSs and MHC class II molecules, precise details at molecular level are not yet clear.[34, 67] In general, peptides are known to bind in a linear, extended conformation and compose of variable length peptide epitopes because of the MHC class II has open ends. ZPSs are required to maintain the dual-charge character, in order to be presented by the MHC class II molecules

and question arises in a way that, does the zwitterionic motif of ZPSs assist in binding to the MHC II complex solely in an electrostatic manner or the dual-charge character is also required to maintain a 3D conformation, allowing the right conformational epitope required for MHC II binding. Using NMR and computational studies, three dimensional structures of two ZPSs, namely Sp1 and PS A2 have been obtained (Figure 4). The 3D conformations of Sp1 and PS A2 ZPSs align with each other very well in a right-handed helix conformation, even though the two polysaccharides have different monosaccharide composition (trisaccharide *vs* tetrasaccharide repeating units). Interesting features observed are equidistantly separated charges (15 Å), eight sugar residues per turn, 20 Å pitch and zig-zag alignment of charges along the helical portion exposed for MHC II and αβ TCR recognition.[67a, 68]

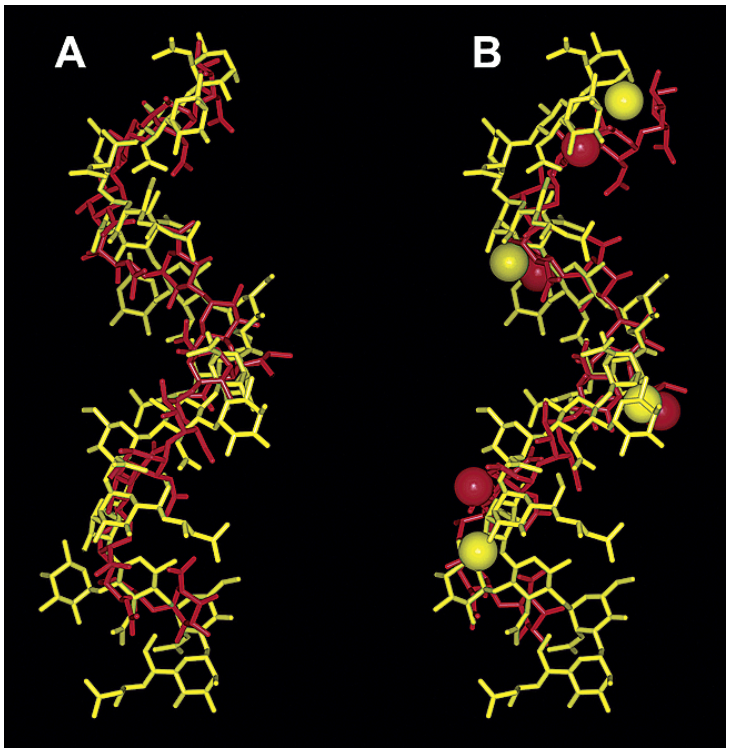


**Figure 1 - 4*:** Superimposed PS A2 and Sp1 polymers.

(Fig 4A: glycosidic oxygen atom-based superimposition; Fig 4B: amino functionality-based superimposition) in yellow and red colors respectively. *"Reproduced with permission from Choi, Y.-H.; Roehrl, M. H.; Kasper, D. L.; Wang, J. Y. *Biochemistry* **2002**, *41*, 15144. Copyright© 2002 American Chemical Society."

### 1.3.2. PS A1 antigen processing and presentation: *T-cell* activation

Polysaccharide A1 (PS A1) is up-taken, processed and presented in a pathway, very much similar to peptide antigens, with a major difference in the way PS A1 is fragmented in the endosomes through reactive nitric oxide radical ($NO^{\cdot}$), in contrast to acidic proteolysis of proteins into smaller peptide epitopes by proteases.[6, 35a] In the simplest way, processing and presentation of PS A1 antigen by APCs is as follows; first, uptake of PS A1 polymer of nearly 110 kDa by APCs through pinocytosis or receptor mediated (DC-SIGN) endocytosis,[69] radical nitric oxide mediated depolymerization of polysaccharide into smaller fragments of 10-15 kDa, fusion of endocytic and exocytic vesicles containing PS A1 and HLA-DR respectively, exchange of PS A1 with CLIP under acidic conditions to form HLA-DR:PS A1 complex and finally presented to αβ TCR of T-cells at the cell surface (Figure 5).

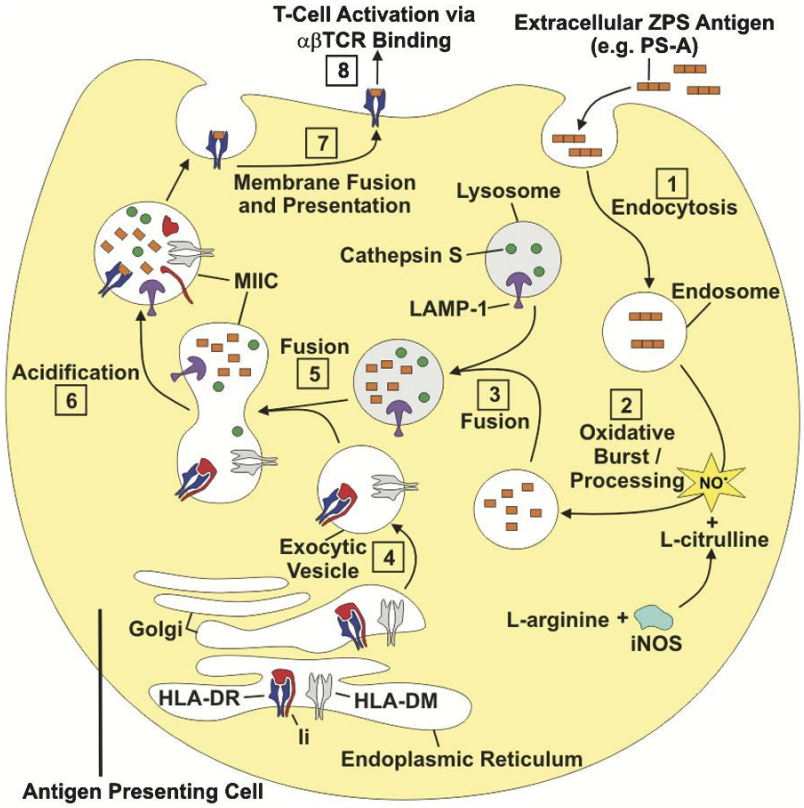


**Figure 1 - 5*:** Polysaccharide A1 (PS A1) uptake, processing and presentation by MHC II in antigen presenting cells (APCs).

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Once PS A1 is endocytosed, inducible nitric oxide synthase (iNOS) catalyzes the oxidation of L-arginine to produce NO· radical, leading to the depolymerization of PS A1 polysaccharide through site-selective deamination of the positively charged amino group in the D-AAT*p* residue of a single repeating unit, resulting in fragments of 10-15 kDa size without effecting the zwitterionic nature of the entire PS A1 antigen.[35b] Using an abscess inducing model in mice, depolymerization of PS A1 by radical nitrogen species was clearly illustrated by using various knockout mice (iNOS$^{-/-}$, NADPH$^{-/-}$ oxidase and using

preprocessed PS A1 fragments) and ruling out the involvement of glycosidases and NADPH oxidase (catalyst for producing radical oxygen species).[35b] Endosomal compartment containing processed PS A1 fragments of nearly 10-15 kDa, exocytic vesicle containing HLA-DR, HLA-DM and lysosomal vesicle fuse together to form MIIC (MHC II containing) vesicle and acidification of the MIIC vesicle leads to HLA-DM catalysis of the exchange of CLIP (a self-peptide binding in MHC II groove to maintain the stability) with processed PS A1 antigen fragments forming MHC II:PS A1 complex. Using an abscess inducing model, chemicals blocking MHC II pathway or blocking HLA-DM in mice have shown the importance of HLA-DM in the catalysis of exchange of CLIP to processed PS A1.[35a, 67b] Confocal microscopy data,[35a] generation of T-cell hybridomas against PS A1 and Sp1[35c, 70] which are cross reactive and co-immunoprecipitation experiments[35b] establish the paradigm shifting carbohydrate mediated activation of the specific subset of T-cells.[6, 71]

### 1.3.2. Studies on MHC II binding with PS A1

Computational studies of PS A2 with MHC II showed that a four-repeating unit (16 sugar residues) PS A2 fragment was able to bind very well with computationally simulated α-helix of MHC II protein (Figure 6).[68] Cobb *et. al* studied the effect of charge and size (MW) on the ability of PS A1 to bind MHC II protein using circular dichroism (CD) experiment. Only positive/negatively charged or neutral PS A1 fragments were unable to bind to MHC II, whereas, zwitterionic fragments within a size range of 3-30 kDa were able to bind to MHC II.[67b] The above study highlighted that a minimum of

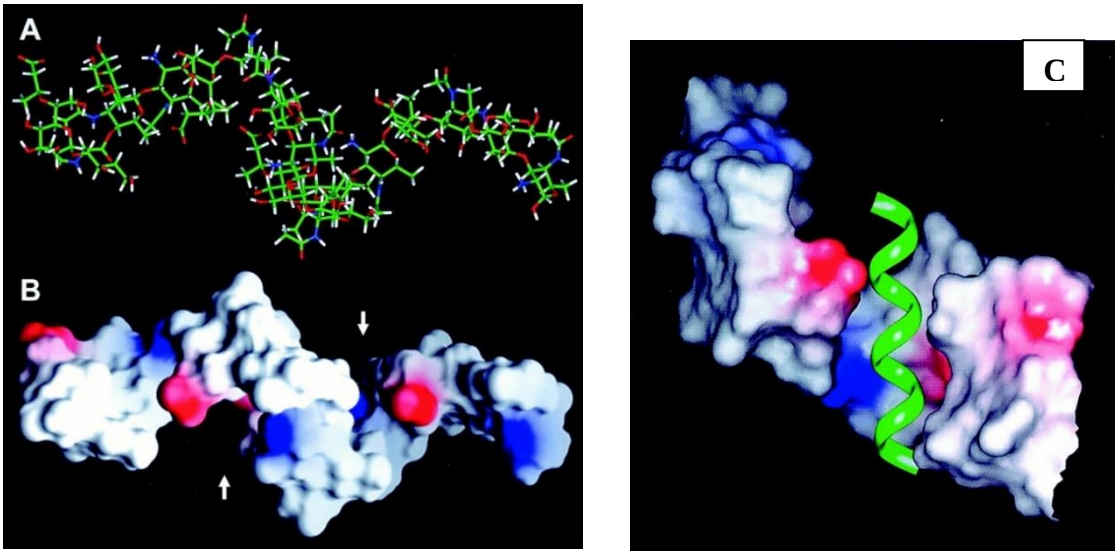



**Figure 1 - 6*:** Stick (Fig 6A) and electrostatic models (Fig 6B) of PS A2 tetramer. Binding model of α-helix with PS A2 tetramer antigen fragment (Fig 6C). *"Reproduced with permission from Wang, Y.; Kalka-Moll, W. M.; Roehrl, M. H.; Kasper, D. L. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 13478. Copyright© 2000 PNAS U. S.A."

three repeating units and zwitterionic charge are extremely important for helical nature of the polysaccharide fragments and binding into the MHC II groove. The zwitterionic charge is important for the helical nature and importantly for the electrostatic binding of PS A1 to MHC II protein. Kasper *et. al* have studied the PS A1 and other proteins (MBPp and SEA) binding with MHC II complex under various concentrations of sodium chloride and have shown the binding affinity of PS A1 was reduced to less than 60% in the presence of NaCl salt solution and whereas other proteins affinity was unaffected.[66] Superantigens are a special class of antigens that activate T-cells by binding outside the MHC II binding pocket, for example, Staphyloccocal enterotoxin A (SEA) is a well-known super antigen. PS A1 ability to bind as superantigen was tested, PS A1 was able to replace MBPp and SEA under

competition experiments with increasing concentrations of PS A1 and it was thought that replacement of SEA might be due to conformational shift in protein structure through PS A1 binding rather than binding outside the binding groove. Various studies also support the fact that PS A1 is not a superantigen.[35b, 35c, 60, 72] PS A1 has higher binding affinity at acidic pH (pH ~ 5.5), has specificity in binding towards MHC II haplotypes and binds to HLA-DR haplotype exclusively. Among the HLA-DR haplotypes, PS A1 binding affinity is in the order of HLA-DR2 with highest affinity followed by HLA-DR4 and HLA-DR1 respectively.[66] Confocal microscopy experiments also highlighted the coexistence of PS A1 antigen and MHC II complex in endosomes, near the cell surface and interaction of the di-localized complex (MHC II:PS A1)with αβ TCR.[35a] From the above experimental data, the precise mechanistic details of how PS A1 binds or fits inside the binding groove is not very clear at molecular level. In order to obtain a clear-cut understanding, a crystal structure of MHC II with a homogenous synthetic fragment would definitely give a direct explanation about the binding process and deciphers the crucial interactions between peptide and sugar residues.

### 1.3.3. Immunomodulatory role of polysaccharide A1 (PS A1):

Even though, *B. fragilis* accounts for only 1% of the total microflora in the human gut, it has great influence over the host physiology (intestinal/extraintestinal). *B. fragilis* was classified into two categories based on the pathogenic/non-pathogenic nature. Pathogenic species producing bacterial toxins are called enterotoxigenic *B. fragilis* (ETBF) and non-pathogenic species that modulate immune responses are called non-toxigenic *B. fragilis* (NTBF).[73] ETBF group of bacteria secrete *B. fragilis* toxin (BFT, metalloprotease

toxin) leading to acute inflammatory disease[74] and is found in higher counts in patients with colon cancer compared to healthy ones.[75] A very interesting study by Sears *et. al* showed that treatment of SPF mice (specific pathogen free) with NTBF *B. fragilis* NCTC 9343 (NTBF 9343) followed by ETBF 86 strain protected mice from colitis and formation of tumors. When NTBF 9343 and ETBF 86 were administered simultaneously or ETBF 86 colonized mice treated later with NCTC 9343, no protection was observed against tumor formation and colitis. The most remarkable outcome from Sears *et. al* work is, specific pathogen free (SPF) mice treated in sequence with PS A1 deficient NCTC 9343 followed by ETBF 86 protected the mice from colitis and tumor development, challenging the immunomodulatory properties of PS A1.[76] However, a significant amount of data strongly supports the immunomodulatory properties of PS A1 and hence reflects the mechanistic details of interaction between bacterial symbiotic molecules and immune cells at a molecular level is not yet clear and needs further experimentation.[77]

*B. fragilis*, when located inside the gut is well-known for its symbiotic nature through the development of immune system, correction of T-cell imbalances and anti-inflammatory properties, mediated through the polysaccharide A1 (PS A1). When *B. fragilis* escapes from the gut into the peritoneum during dysbiosis (physical trauma or surgery), it causes severe abscess formation due to activation of pro-inflammatory Th17 cells.[48a] Treatment of mice with live *B. fragilis*, heat killed *B. fragilis*, pure CPC or PS A1 polysaccharide along with sterile cecal contents led to abscess formation, whereas unencapsulated *B. fragilis* failed to induce the formation of abscess.[49a] So, based on the physiological context of *B. fragilis* presence, the nature of pro-inflammatory *vs* anti-inflammatory response was observed. Wang *et. al* proposed a model for the induction of

abscess by *B. fragilis* in the peritoneum based on the experimental results (Figure 7), PS A1 binds to TLR-2 (Toll-like receptor), activating the MyD88 pathway, leading to the

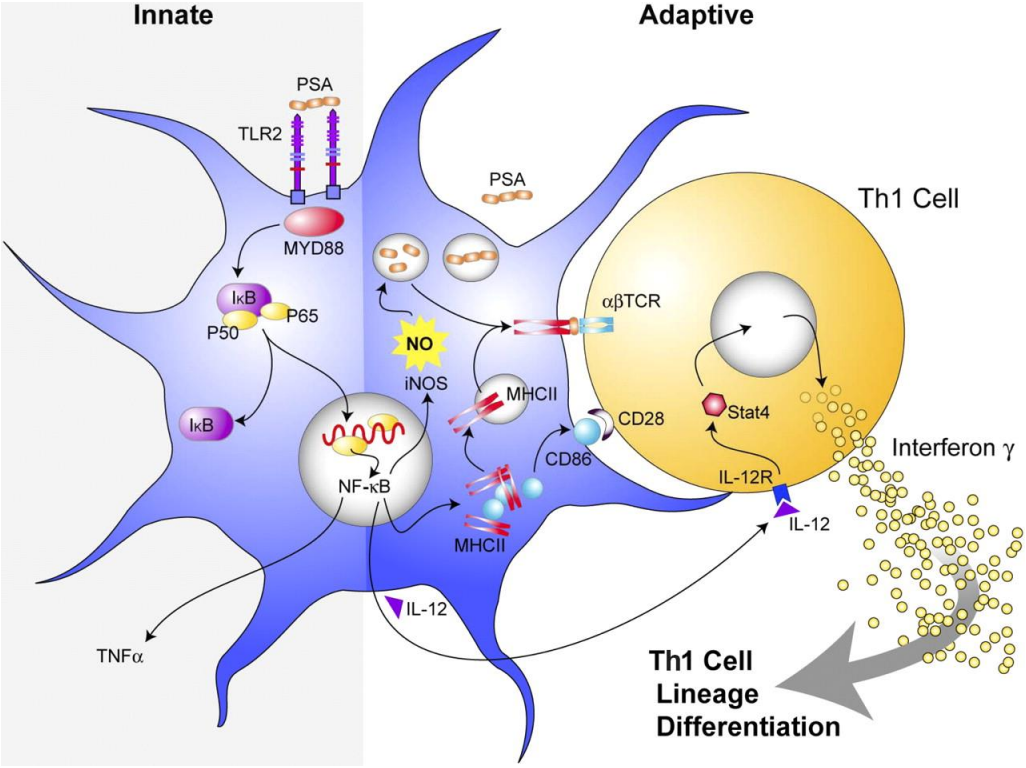



**Figure 1 - 7*:** PS A1 mediated abscess formation model through innate and adaptive arms of the immune system. *"Reproduced with permission from Wang, Q.; McLoughlin, R. M.; Cobb, B. A.; Charrel-Dennis, M.; Zaleski, K. J.; Golenbock, D.; Tzianabos, A. O.; Kasper, D. L. *J. Exp. Med.* **2006**, *203*, 2853. Copyright© 2006 Rockefeller University Press."

translocation of the transcription factor NF-κB into the nucleus of the antigen presenting cells (APCs). The translocation of NF-κB leads to the transcription of genes required for the secretion of TNF-α/IL-12, enhanced expression of radical nitric oxide producing enzyme iNOS and up-regulation of proteins like MHC-II/CD86. Presentation of the

processed PS A1 by MHC II and in combination with IL-12 production (TLR-2 mediated) by APCs leads to the activation of $CD4^+$ T-cells and differentiation into Th1 cells secreting pro-inflammatory cytokine IFN-γ. TLR-2 connects innate and adaptive arms of the immune system through PS A1 binding and mediates the formation of abscess in mice models.[60]

*B. fragilis* exerts a great deal of symbiotic beneficiary properties when present in its natural habitat, the gut. Symbiotic properties of *B. fragilis* are mediated through the CPC, especially PS A1.[6] In general, germ free mice (GF mice) have very high number of Th2 cells compared to Th1 cells, can lead to potential risk to the normal health of an individual causing various inflammatory, allergic and infectious diseases.[78] *B. fragilis* expressed PS A1 is the first known commensal symbiotic factor that can correct the abnormal ratio of Th1/Th2 cells, lymphoid follicle growth defects, leading to a healthy balance of the immune system.[61] The ability of *B. fragilis* to colonize the gut requires the suppression or control of IL-17 secreting Th17 cells, reflecting the induction of anti-inflammatory response mediated by PS A1. GF mice monocolonized with PS A1 expressing *B. fragilis* have lower number of Th17 cells compared to the mice colonized with PS A1 deficient mutant bacteria.[79] Protective effects of PS A1 were studied using colitis inducing T-cell transfer ($CD4^+$ $CD45Rb^{high}$) model and colonization of colitis inducing bacterium *Helicobacter hepaticus*,[80] *Bartonella henselae* mice models.[81] The mice colonized with wild-type *B. fragilis* (WT *B. fragilis*) or administration of PS A1 orally lead to the protection from colitis, similar results were observed with chemically induced model of colitis [trinitrobenzenesulfonic acid, TNBS]. PS A1 anti-inflammatory nature protects from necrotizing enterocolitis through suppression of IL-8 production,

colonization of various pathogenic bacteria like *Vibrio parahaemolyticus* (gastroenteritis)[82] and lower counts of *B. fragilis* can lead to increased count of pathogenic bacteria like diarrhea inducing *Clostridium difficile*[83] and *Clostridium perfringens*.[84] The anti-inflammatory properties of PS A1 are mediated by IL-10 through the activation of a specific set of $CD4^+$ $CD25^+$ $Foxp3^+$ T-regulatory cells.[52b] Recent study by Dasgupta *et. al* reported that PS A1 mediated immunomodulation is dependent upon a specific type of tolerogenic plasmacytoid dendritic cells (pDCs) rather than the conventional dendritic cells (cDCs). pDCs activate Treg cells in a more potent way compared to cDCs *in vitro* and in chemically induced colitis models (TNBS), transfer of PS A1 pre-incubated pDCs offered more protection than the conventional DCs and the protection is dependent on pDCs count.[63]

Immunomodulation by polysaccharide A1 (PS A1) is not just confined to the gut, PS A1 can protect from various types of extraintestinal diseases like allergy, asthma, *Bartonella henselae* induced tissue damage and experimental autoimmune encephalomyelitis (EAE).[77, 85] Extraintestinal protective ability of PS A1 is also mediated by the anti-inflammatory cytokine IL-10. All the above protective properties both inside the gut and extra intestinally reflect the ability of using PS A1 as a prophylactic and therapeutic agent (Figure 8). In EAE mouse model, colonization of PS A1 expressing *B. fragilis* species protected the mice after the antibiotic treatment, whereas the PS A1 lacking *B. fragilis* failed to offer the protection and protective properties were mediated by the anti-inflammatory cytokine IL-10 producing $Foxp3^+$ T-cells.[86] Importance of PS A1 expression was further validated by the two mice models of asthma[62] and *Bartonella henselae* mediated tissue damage,[87] PS A1 protected the mice from asthma and tissue damage

through pDCs activated Tregs secreting IL-10. All the above results explain the importance of the microbiota or symbiotic bacteria specifically to maintain the immune homeostasis and the detailed mechanistic details of how they do it is still not very clear.

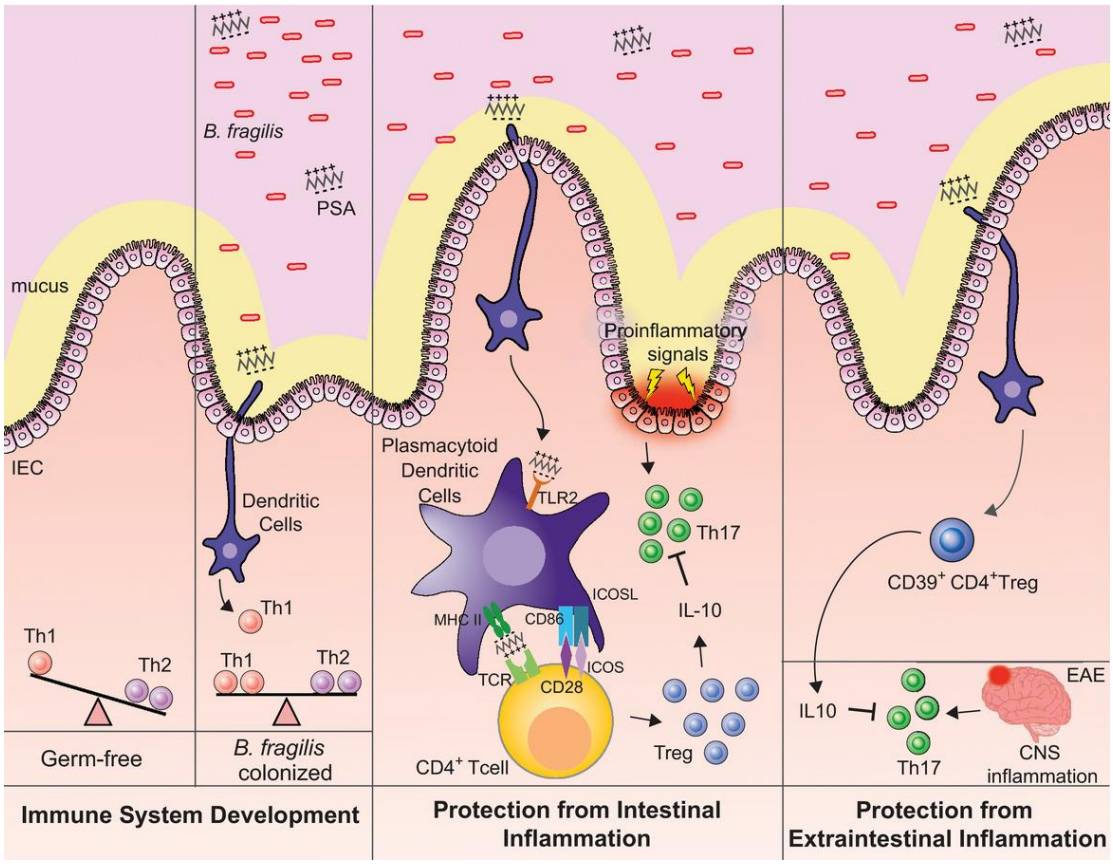



**Figure 1 - 8*:** PS A1, symbiotic factor from *B. fragilis* mediated immunomodulation of the immune system in mice model. *"Reproduced with permission from Erturk-Hasdemir, D.; Kasper, D. L. *Ann. N.Y. Acad. Sci.* **2018**, *1417*, 116. Copyright© 2018 The New York Academy of Sciences."

Further experimentation is clearly required to validate the specific symbiotic factors secreted by various symbiotic bacteria involved in the process of immunomodulation, both in the gastrointestinal tract (GI tract) and extraintestinal organs. PS A1 is known to interact with TLR-2 in the gut to produce majorly an anti-inflammatory response, whereas in the

peritoneum leads to a pro-inflammatory response leading to the formation of abscess.[77] DC-SIGN, a C-type lectin is known to bind to PS A1 in the process of uptake[69] into the APCs and TLR-2 is not necessary for the uptake of PS A1.[60] Overall, expect TLR-2 and DC-SIGN interacting with PS A1 and specific MHC-II haplotype HLA-DR presenting the antigen to TCR of T-cells, different other receptors interacting PS A1 and various signaling pathways being initiated by PS A1 need to be understood yet.

Based on the MHC II mediated T-cell activation, PS A1 was used as a carrier of various tumor associated carbohydrate antigens (TACAs) instead of immunogenic proteins, to develop an entirely carbohydrate-based vaccine. The major focus of our research group for the past 14 years was to target various cancer antigens using PS A1 conjugated TACAs and very promising results have been observed for developing entirely carbohydrate-based vaccines which will be discussed in the next section.

### 1.4. Entirely carbohydrate-based vaccines: an *optimistic* alternative to highly immunogenic carrier proteins

The idea of vaccine was discovered by Edward Jenner in the late 18$^{th}$ century while working with small pox virus,[88] since then, vaccines have been protecting millions of children, adults and the elderly until now from highly pathogenic bacteria, virus and fungi.[89] Various glycoconjugate vaccines are available in market today to fight various bacterial diseases like PedvaxHIB®, Hiberix®, Menjugate® and Prevnar 13®.[90] A limited number of pure capsular polysaccharide vaccines are also available in market today like *Neisseria meningitides* vaccine,[91] Vi vaccine[92] and Pneumovax® 23.[93] Capsular polysaccharides of bacteria are made up of hundreds of repeating units, consisting of

monosaccharides varying in ring conformation, stereospecific glycosidic linkages and are considered to evoke T-cell mediated immune response. The lack of T-cell activating ability of polysaccharides led to the idea of glycoconjugate vaccine strategy, in which the capsular polysaccharide antigen fragments are conjugated to immunogenic proteins and hence lead to T-cell activation.[6] Even though conjugation to proteins help in the generation of highly specific antibodies and memory cells towards the carbohydrate antigens, due to the high immunogenic nature of proteins most of the antibodies generated are specific towards the carrier protein, a phenomenon called as carrier induced epitope suppression.[6, 9] Recently, zwitterionic polysaccharides (ZPSs) are known to elicit T-cell dependent immune response in the absence of proteins and hence open a novel platform to target various bacterial and cancer antigens avoiding the highly immunogenic protein carriers.[9] Based on the various path breaking scientific outcomes, cancer cells differ from the normal healthy cells in the aberrant glycosylation pattern and various tumor associated carbohydrate antigens (TACAs) like mucin-type, glycosphingolipids and Lewis blood group type have been identified.[94] Thomsen-nouveau (Tn), Thomsen-Friedenreich (TF), sialyl Tn (STn) come under mucin type *O*-antigens. LewisX ($Le^x$), LewisY ($Le^y$), LewisA ($Le^y$), sialyl LewisA ($sLe^a$), sialyl LewisX ($sLe^x$) and others belong to blood group antigens. TACAs related to glycosphingolipids are GM2, Globo H, GD2, GD3 and others. Even though significant research on cancer cells led to great level of understanding at the molecular level, until now no cancer vaccine is available in the market except Provenge®.[95] The reason for the failure in developing an effective cancer vaccine is the self-tolerant property of TACAs and immune suppressive mechanisms associated with cancer cells.[96] Conjugation of TACAs to protein carriers and immunological studies were carried into clinical trials, but none of

those conjugates were successful as expected.[97] Our group has been focused on the development of entirely carbohydrate-based vaccines targeting cancer, using the ZPS PS A1 as a carrier for various TACAs.[98]

The zwitterionic tetrasaccharide repeating unit of PS A1 is composed of four monosaccharide repeating units, namely, D-Gal*f*, D-GalNAc, D-AAT and pyruvate D-Gal*p* with positive and negative charges on D-AAT and pyruvate D-Gal*p*, respectively. Chemo-selective oxidation of terminal vicinal diols in D-Gal*f* residue using sodium periodate ($NaIO_4$) afforded oxidized PS A1 with active aldehyde functionality (Figure 9). Aminoxy derivatives (reducing end) of various TACAs synthesized were conjugated to the oxidized PS A1 to attain TACA:PS A1 conjugates linked through an oxime.[98c, 99] Various *O*-glycosylated alkyl amine linkers installed at the reducing end are known in the literature for the conjugation of a glycoantigen to a protein, with ZPSs like PS A1 and Sp1, amine-based linkers cannot be used due to the presence of primary amine group in the repeating unit itself.[100]

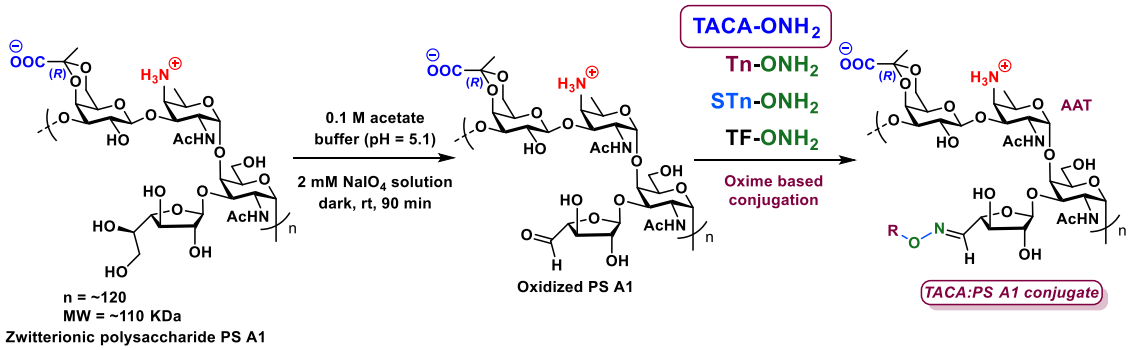



**Figure 1 - 9:** Conjugation of TACAs to oxidized PS A1

Various TACAs like Tn,[99a] TF[98c] and STn[98a] were conjugated to PS A1 and studied in mice. Immunological studies with TACA:PS A1 conjugates in C57BL/6J mice models gave promising results with the generation of highly specific antibodies (IgM and IgG)

targeting specifically TACA expressing cancer cell lines. All the above results provide an optimistic alternative to the current glycoconjugate vaccines to develop an entirely carbohydrate-based vaccine in the near future targeting various cancers.

### 1.5. Importance of chemical synthesis: PS A1 repeating units

Polysaccharide A1 (PS A1) is isolated from the *B. fragilis* bacterial culture through a tedious and laborious multi-step purification protocol[99a] with very low yield. Furthermore, the most important limitation of using biologically isolated PS A1 in studying the mechanistic details of T-cell activation at a molecular level is its heterogeneous nature (polymers with varying kDa size). To understand the mechanistic details of antigen uptake (various receptors involved), processing and presentation of PS A1 in the context of MHC II by the antigen presenting cells (APCs), structurally well characterized or defined moieties are of very high importance. To obtain structurally defined antigen fragments of PS A1 for the mechanistic studies, a well-thought and executed synthetic route can provide PS A1 synthetic fragments in sufficient quantities within a specific time. PS A1 can be used as a carrier not only for TACAs but also for various other bacterial antigens[101] to develop vaccines to encounter various pathogenic bacteria. Vaccines based on the synthetic oligosaccharide antigen can solve various problems related to traditional vaccine synthesis involving culture of bacteria, as all species of bacteria are not able to culture in huge quantities,[102] tedious purification steps sustaining the CPS (few strains of CPS decompose) and quality control factors like batch to batch reproducibility and chemical analysis can lead to enormous rise in the cost of vaccine per dose.[103] Compared to the human glycome, the bacterial glycome is very diverse and complex. It consists of various

rare sugar motifs connected in a very specific fashion (regio and stereospecific) through glycosidic linkages and posing further challenge to chemical synthesis.[104] The key factor in the development of efficient vaccine candidate against a bacterial disease is to find the immunologically protective epitope (protective epitope). Studies on PS A1 polysaccharide and chemically processed fragments (ozonolysis) showcase the importance of size of the PS A1 antigen in kDa *vs* the observed immunological outcome. The minimum size of PS A1 antigen fragment to have helical nature is 3 kDa and the bioactivity of pre-processed fragments of PS A1 with 15-20 kDa is as efficient as 110 kDa polymer.[105] Up until now, none of the synthetic repeating units of PS A1 and Sp1 have been tested *in vivo*, resulting in the lack of information regarding the effect of size on T-cell activation phenomenon. As *B. fragilis* ATCC 25285/NCTC 9343 is a symbiont rather than a pathogen, the determination of the right epitope (*sequential* or *conformational*) of PS A1, which can be an efficient activator of T-cells will help in developing completely synthetic vaccine candidates targeting various bacterial diseases and carcinoma.

### 1.5.1. Immunologically active epitope determination: PS A1

Over the past few decades, the process of determination of the right/protective epitope has been iterative. The carbohydrate antigen is synthesized based on the repeating unit of the CPS, conjugated to a carrier protein, immunologically evaluated and if the results are not satisfactory, a new antigenic motif is designed and tested.[103] In the context of polysaccharide A1 (PS A1), the determination of the right epitope refers to finding the synthetic fragment (disaccharide to multi-repeating unit) that can efficiently activate the $CD4^+$ T-cells. Based on the previous work of Kasper and others, a minimum of 3 kDa is

required to form a helical structure, processed PS A1 fragments between 3 to 10 kDa showed better binding ability to MHC II protein and in the mice abscess protection model, nearly 17 kDa PS A1 fragment was as effective as the entire 110 kDa polymer based on both *in vivo* and *in vitro* studies and of all the structural parameters zwitterionic charge was the most crucial. Tn antigen was conjugated to PS A1 to obtain Tn-PS A1 conjugate and circular dichroism experiments[106] at various pH showed a decrease in α-helicity of conjugate (around pH 3.8 - 8.4) to complete loss of helicity (pH < 3.5 and pH > 8.5), a result contrast to previous work,[35a, 67b] as Tn-PS A1 conjugate elicited T-cell based response. Based on the above results, the endosomal and exocytic vesicles fuse to form MIIC vesicle containing HLA-DR with CLIP, HLA-DM and processed PS A1 fragments, and the acidic pH (around pH ~ 5) will lead to the protonation of the amine residue in D-AAT and is crucial to form a helical structure that leads to efficient binding with MHC II (HLA-DR) complex. Compiling all the data available regarding the PS A1, various structural parameters like size of the repeating unit, frameshift or sequence, helical nature and zwitterionic charge might be crucial in the T-cell activation process. The idea of *sequential* and *conformational* epitopes was developed by Arnon *et. al*,[107] *sequential* epitope refers to the frameshift or order in which protein/sugar residues are arranged in the primary structure and *conformational* epitope refers to the recognition process of specific set of residues in close proximity because of three-dimensional structural arrangement. For example, group B streptococcus (GBS) type III antigens should have a minimal size of five repeating sequences in order to be immunologically active (*conformational* epitope), whereas, others like *S. pneumoniae*, Hib or *Shigella* require smaller to medium epitope length for immune recognition (*sequential* epitopes).[103] In regards to PS A1, the question

becomes, is it the sequence or the conformation that matters, or both sequence and conformation, needs to be further elucidated? The question can be answered by synthesizing PS A1 monomeric repeating unit; synthesis containing all the three possible sequences or frameshifts and antigenic fragments of higher molecular weight (>3 kDa), and followed by *in vitro* and *in vivo* studies (Figure 10).

The hypothetical model for PS A1 mediated activation of T-cell is shown below (Figure 11), HLA-DR haplotype is the MHC II protein that processed PS A1 fragments bind and are presented to the αβ TCR of $CD4^+$ T-cells. In the model shown below, the alpha and beta chains of HLA-DR2 haplotype are represented in blue and pink colors respectively. Two modes of PS A1 dependent activation of $CD4^+$ T-cells might be possible, PS A1 fragments of higher molecular weight (> 3 kDa) can attain a helical conformation, exposing the positively charged amine functionality in the D-AAT residue

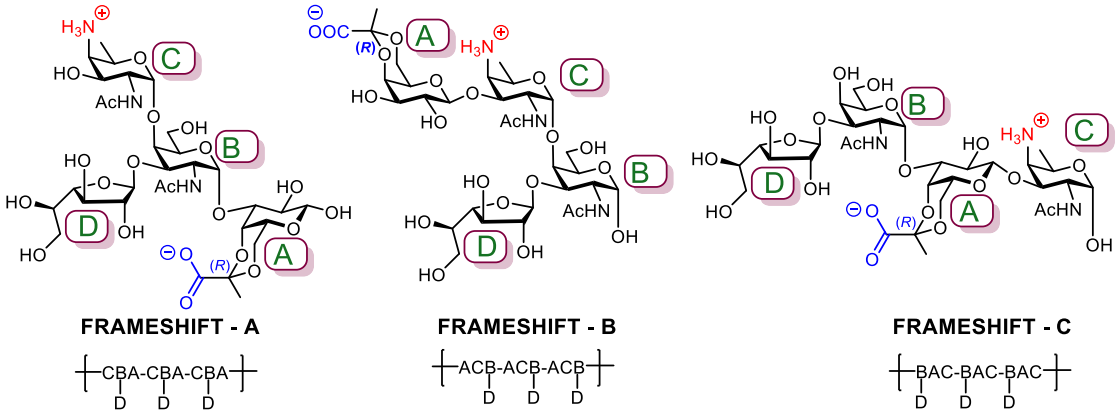



**Figure 1 - 10:** Three different sequential epitopes for PS A1 repeating unit (frameshift **A**, **B** and **C**).

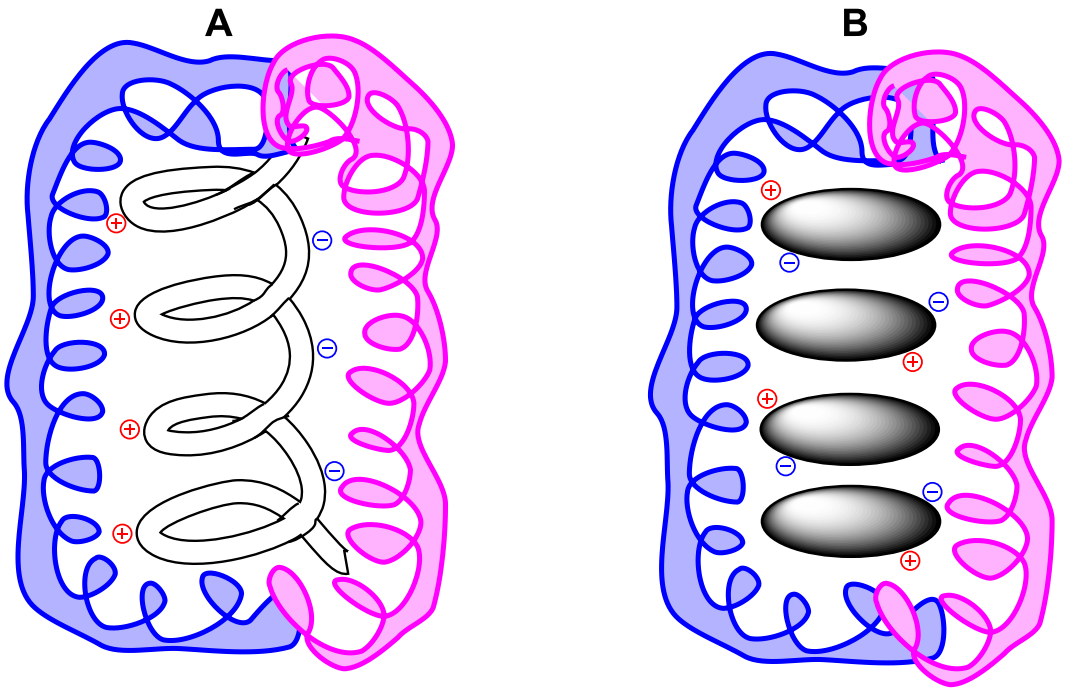



**Figure 1 - 11:** Hypothetical binding model of synthetic PS A1 fragments with MHC II [HLA-DR2] protein in the binding groove. Top view of PS A1 antigen fragment(s) in the binding pocket of HLA-DR2 protein. Alpha and beta chains of HLA-DR2 heterodimer drawn in blue and pink colors respectively. Figure **A**: Helical PS A1 fragments of higher molecular weight (> 3 kDa) with alternating charges on opposite ends interacting electrostatically with peptide residues [*conformational* epitope]. Figure **B**: zwitterionic, single repeating units of PS A1 interacting in an individual block-type fashion [*sequential epitope*].

and negatively charged carboxylate functionality in the pyruvate D-Gal*p* residue on the opposite sides of the helical antigenic fragment, and thus positioning the charged residues in the right place for the electrostatic interaction with the acidic and basic peptide residues in the binding groove of the HLA-DR2 complex (Figure 11, A). The above hypothesis can

be put to test through gaining access to zwitterionic PS A1 antigen fragments of molecular weight greater than 3 kDa and attempting *in vitro* and *in vivo* studies to confirm the process of T-cell activation. The zwitterionic, single repeating unit sequences might bind in the binding groove in a block-type fashion interacting with various acidic and basic peptide residues, mainly through electrostatic interaction, and thus activating the T-cells (Figure 11, B). The above hypothesis can be tested in mice models using the synthetic *sequential* antigenic repeating units (frameshifts A, B and C) and PS A1 antigenic fragments of higher molecular weight (> 3 kDa, *conformational* epitopes).

The general size of antigen fragments that bind to the MHC II complex in the binding grove are known to be around 1.6 – 3.2 kDa for peptide fragments and it is also known that MHC II complex has open ends leading to the size independent nature of the antigen fragments that bind to the MHC II and are presented to the TCR complex of the $CD4^+$ T-cells. Based on the previous studies conducted by Kasper group, the effect of molecular size on the binding to the MHC II complex was studied using ELISA technique and was shown that antigen fragments with less than three repeating units failed to bind the MHC II complex. Even though, *in vivo* studies using these molecular fragments with molecular weight lower than the 3 kDa was not attempted, hence the information related to the size dependent activation of the $CD4^+$ T-cell activation remains unknown. The synthesis of three sequential epitopes and antigen fragments of higher molecular weight (> 3 kDa), followed by *in vitro* and *in vivo* studies is of very high importance to know the effect of sequence and size of the PS A1 antigen fragments on the T-cell activation. Successful determination of the active or right epitope of polysaccharide A1 (PS A1) will help to develop an entirely synthetic carbohydrate-based vaccine to target various

pathogens by conjugating the right epitopes of virulent pathogens to the synthetic PS A1 carrier and also various TACAs can be conjugated in a chemo-selective fashion to fight cancer.

### 1.5.2. Future directions:

The determination of the immunologically active epitope of PS A1 has significant importance. To understand the precise molecular details involved in the paradigm shifting phenomenon of T-cell activation by the zwitterionic polysaccharides (ZPSs), completely defined synthetic PS A1 antigenic fragments are of high importance. PS A1 is known to link innate and adaptive immune systems through TLR2 receptor and DC-SIGN is also known to assist in the PS A1 antigen uptake, through a receptor mediated process. Other than the above mentioned two receptors, very less information is known regarding the various receptors that can interact with PS A1. Synthetic and structurally defined PS A1 antigenic fragments can be used as molecular probes to understand the critical details involved in the uptake, processing and activation of T-cells mediated by the APCs.

Synthetic PS A1 active epitope can help in the development of completely synthetic vaccine constructs, solving various quality-control issues like percent loading, reproducibility and structure characterization, and thus leads to low cost per dosage of the vaccine. Various pathogenic bacterial epitopes and various TACAs can be conjugated to the PS A1 synthetic fragment in a chemo-selective fashion, completely defined vaccine constructs can be generated and using structure-activity relationship studies, the efficacy of the vaccine constructs can be tuned to the maximum. Using synthetic fragments of PS A1, vaccine constructs can be developed in a complete rational fashion, in contrast to the

trial and error process. PS A1 synthetic fragments can also be used as an adjuvant, based on the activity, can be structurally modified through chemical synthesis to develop more efficient synthetic immune system activators. Activation of $CD4^+$ T-cells by PS A1 is a well-established fact, but, the critical interactions responsible for the binding in the MHC II groove and recognition by the αβ TCR of the T-cells is not well understood at a molecular level. Using the synthetic PS A1 probes, MHC II protein and PS A1 antigen interactions can be studied using different techniques like isothermal titration calorimetry (ITC), surface plasmon resonance (SPR) and the real breakthrough can be achieved by obtaining the crystal structures of the di-localized complex of PS A1:MHC II and even tri-localized complex of MHC II:PS A1:αβTCR. Crystal structure of the afore mentioned di-localized and tri-localized complex can reveal a great deal of information in detail and the various types of interactions involved between peptide and sugar residues in the MHC II binding groove and αβ TCR can be understood at a molecular level. Hence, the role of chemical synthesis in understanding the immunomodulatory properties of PS A1 is of utmost importance, as access to defined homogenous PS A1 fragments through bacterial culture is not possible as per today.

# Chapter 2

# Total Synthesis of Zwitterionic Repeating Unit of Polysaccharide A1 (PS A1) with Alternating Charges on Adjacent Monosaccharides from Bacteroides fragilis ATCC 25285/NCTC 9343

## 2.1. Introduction:

Zwitterionic capsular polysaccharides (ZPSs), a special class of carbohydrate antigens with T-cell activation capability has been well-studied[6, 58, 77] and used as a carrier to develop a novel class of entirely carbohydrate-based vaccines.[98a, 98c, 99a, 101] The unique T-cell dependent property is directly correlated to the structure of these T-cell activating ZPSs, with positively charged amino groups, negatively charged pyruvate or phosphate groups present in the repeating unit sequence, having helical 3D structure with positive and negative charges aligned on the opposite sides and separated equidistantly. Apart from the dual or zwitterionic charge nature, the repeating unit composition is rich with highly immunodominant sugar residues like D-GalNAc, D-Gal*f*, pyruvate D-Gal*p*, D-GalA and D-AAT.[6, 108] D-AAT (2-acetamido-4-amino-2,4,6-trideoxy-D-Gal) is a rare sugar residue found in various bacteria connected to various other sugar residues in a stereospecific manner that vary based on the type of bacterial species, not present in the human glycome

and offer a chance to develop various bacterial vaccines.[104, 109] ZPSs lose the ability to activate the T-cells, if the charge residues are modified chemically into neutral functionalities like acetamide or ester. Many different research groups have up taken the challenging synthetic endeavor of these ZPSs, to gain access to synthetic repeating unit(s) and study the T-cell activation mechanism in detail.[109] Taking a look at the brief history of previously accomplished synthesis of ZPS repeating unit(s) (Figure 12), Bundle *et. al* synthesized the monomeric and dimeric repeating units of *Streptococcus pneumoniae* type 1 (Sp1) in 2010 and until now is the ZPS synthetic fragment with more than one repeating unit. Unfortunately, none of the repeating unit sequences were capable of activating T-cells *in vitro*, and due to the synthetic challenge associated with the rare sugars connected in a 1,2-*cis* fashion, further synthesis to access Sp1 fragments of more than two repeating units was not up taken.[110] In 2011, Codee *et. al* synthesized all three possible repeating unit sequences of Sp1 using GalA 3,6-synthon strategy[111] and through iterative approach by others.[112] In 2007, van den Bos *et. al* synthesized the completely protected PS A1 repeating unit,[113] followed by Seeberger *et. al* accomplishing the first total synthesis of completely deprotected repeating unit of PS A1 with zwitterionic charges separated by neutral D-GalNAc residue.[114] In 2014, Seeberger *et. al* accomplished the synthesis of conjugation-ready Sp1 and PS A1 single repeating units with active thiol linkers at the reducing end for the ability to conjugate these synthetic probes to an array or immunogenic carrier proteins.[100] From the immunological results published, Sp1 monomeric unit was shown to be recognized by the antisera of the Sp1 polymer, whereas PS A1 monomeric unit was not recognized by the antisera of *B. fragilis*. Very recently, Troutman *et. al* accomplished the biosynthesis of PS A1 repeating unit.[115] Our group completed the first

total synthesis of zwitterionic PS A1 repeating unit with alternating charges on the adjacent monosaccharides, a structural feature we believe to be critical for the immunological activity.[33] Apart from Sp1 and PS A1 ZPSs synthesis, other bacterial ZPS repeating unit synthesis have been accomplished recently by various research groups. Mulard *et. al* and Kulkarni *et. al* accomplished the synthesis of *Shigella sonnei* trisaccharide repeating unit[116] and phosphorylated trisaccharide repeating unit (one-pot) of *Providencia alcalifaciens* O22 respectively.[117] *Staphylococcus aureus* capsular polysaccharides CP5 and CP8 are also zwitterionic, the non-zwitterionic versions of CP5 and CP8 repeating units have been accomplished by Demchenko *et. al*, Boons *et. al* and Adamo *et. al*.[118]

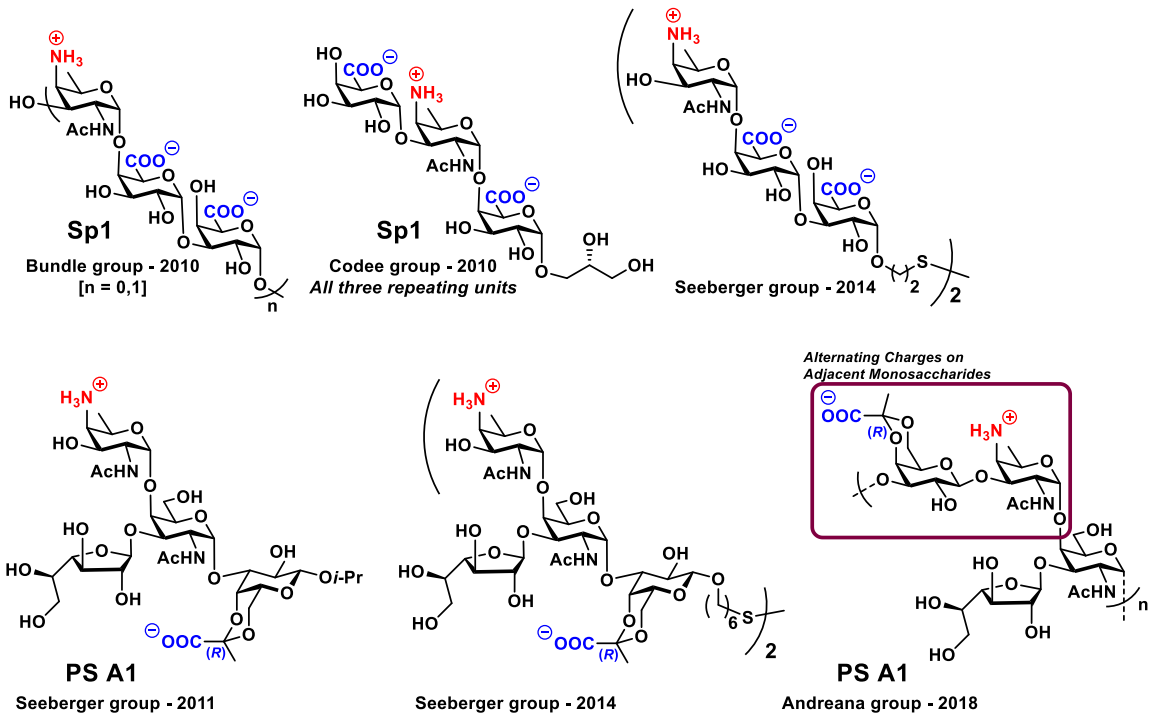



**Figure 2 - 1:** Previous synthesis of various zwitterionic repeating units (ZPSs)

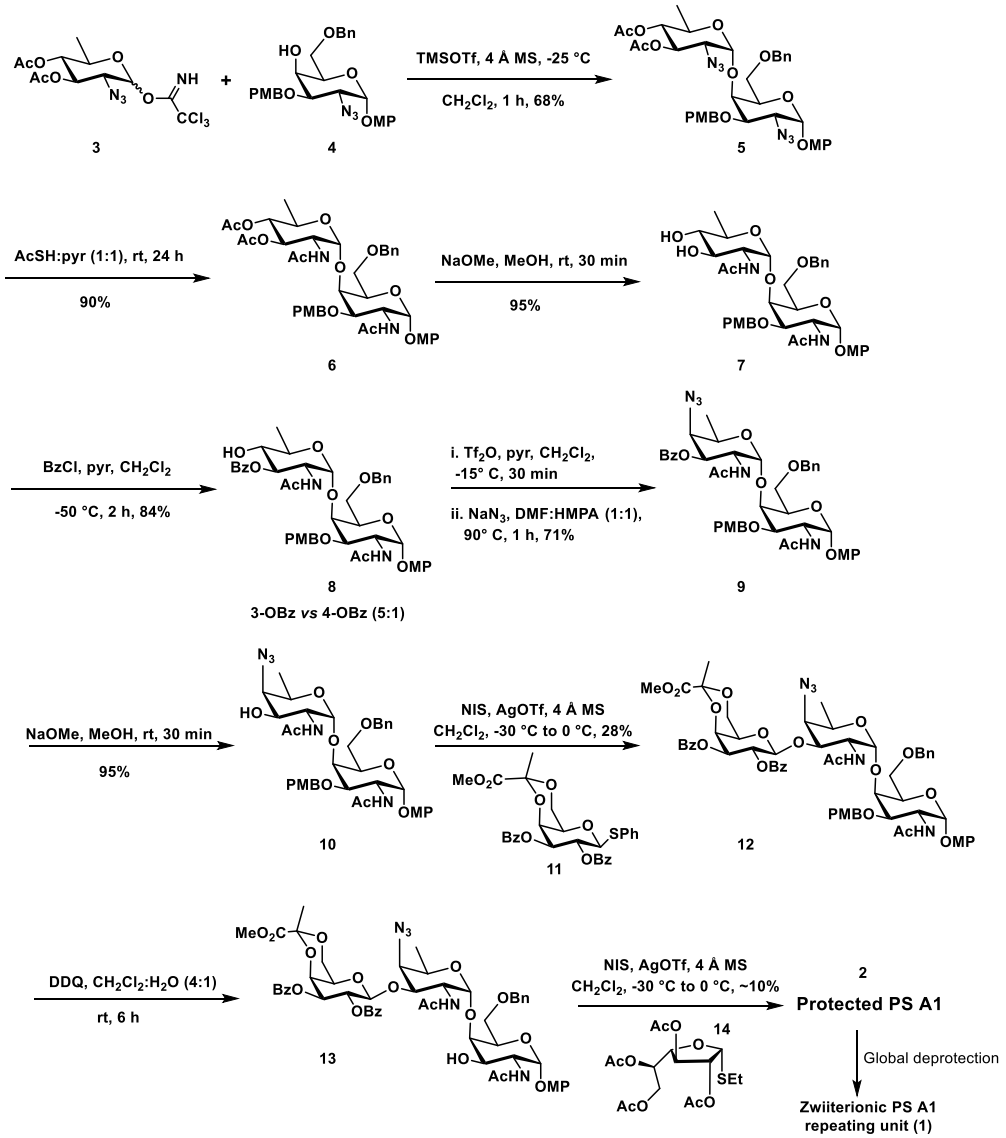
In the last two decades, the knowledge of synthetic carbohydrate chemistry has increased tremendously, various challenging synthesis have been accomplished using one-pot, pre-activation protocol,[119] automated assembly[120] and convergent synthetic strategies.

At present, our understanding in regards to various key aspects of chemical glycosylation like, reactivities of various glycosyl donor and acceptors, effect of protecting groups, conformation, remote participation and other factors has increased way better than the previous decade, and yet a plenty of vigorous research is required to understand various other aspects of oligosaccharide synthesis to expedite the current pace.[121]

### 2.2. Initial attempt(s) towards the PS A1 zwitterionic repeating unit synthesis:

A linear glycosylation strategy was followed to access the zwitterionic repeating unit **1** of PS A1, starting with [1+1] glycosylation of glycosyl donor **3** and acceptor **4** to afford the desired disaccharide **5** in 68% yield.[122] The respective disaccharide **5** was subjected to one-pot azide to acetamide reduction conditions using thioacetic acid (AcSH) and pyridine (pyr) at room temperature to afford the desired diacetamido disaccharide **6** in 90% yield.[123] Zemplén deprotection of two acetate groups in compound **6** afforded the desired diol compound **7** in 95% yield.[124] Selective benzoylation of the diol **7** at – 50 ˚C using benzoyl chloride (BzCl) and pyridine in dichloromethane (DCM) led to the formation of regiomers C3 *vs* C4 hydroxyl protected compounds **8** and **8a** (5:1) in 84% yield.[110] C4-OH alcohol **8** was subjected to one-pot triflate protection and inversion with sodium azide ($NaN_3$) and the desired azide **9** was obtained in 71% yield over two-steps.[104] Zemplén deprotection of the compound **9** led to the formation of C3-OH disaccharide acceptor **10**. Using a [2+1] glycosylation strategy, the disaccharide acceptor **10** and the pyruvate D-Gal*p* donor **11** were subjected to various glycosylation conditions like NIS/TMSOTf, MeOTf, DMTST and NIS/AgOTf, only NIS/AgOTf mediated glycosylation afforded the desired trisaccharide **12** in a low yield of 28% (Scheme 1).[110] PMB protecting group was oxidatively cleaved using DDQ and the desired trisaccharide acceptor **13** was obtained in

69% yield.[124] Final [3+1] glycosylation was attempted with D-Gal*f* donor **14** under various glycosylation conditions like NIS/TMSOTf, MeOTf, DMTST and NIS/AgOTf, desired protected tetrasaccharide **2** was obtained in ~10% yield with NIS/AgOTf activation protocol. The final two glycosylation reactions were very low yielding for the following reasons (Scheme 1).



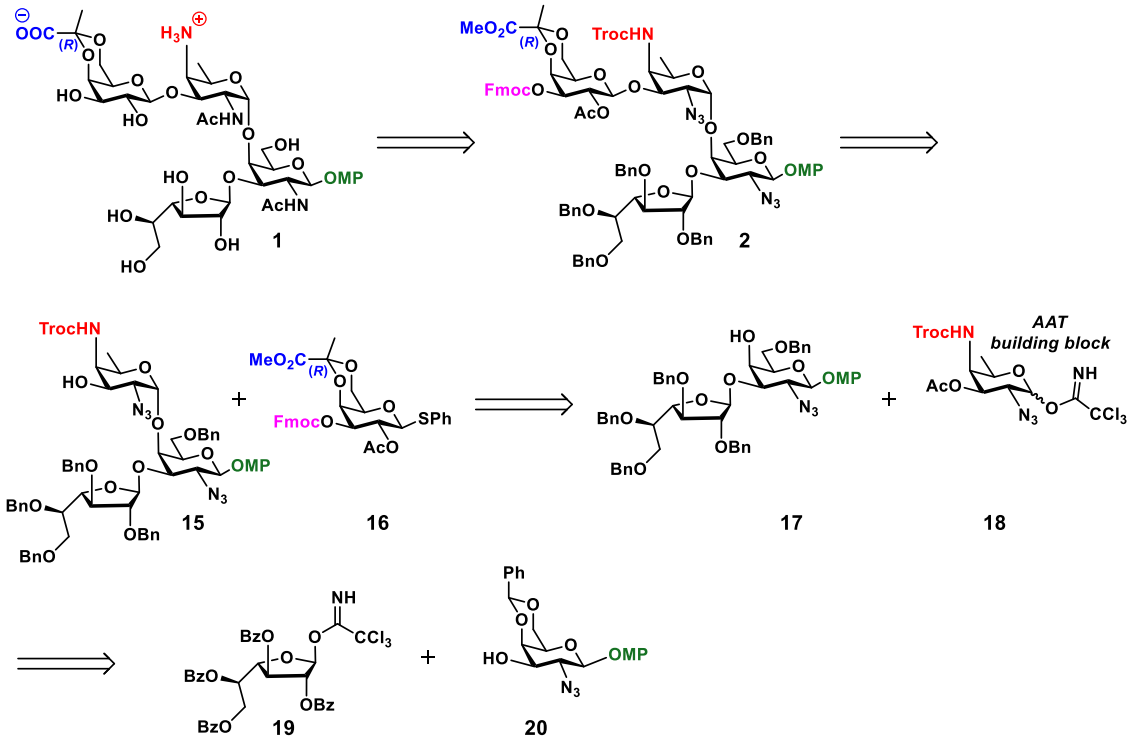

**Scheme 1:** Initial attempt towards the oligosaccharide assembly of PS A1 tetrasaccharide repeating unit **1**

The very low solubility of the diacetamido di and trisaccharide acceptors in dichloromethane at lower temperatures below -20 ˚C and the low reactivity of the diacetamido acceptors (H-bonding with OH) and disarmed nature of the donors used. Acetamide functional acceptors were known to be used previously in the literature, have worked successfully in some cases[110] and shown poor reactivity in other cases.[125] As glycosylation is the key reaction in the process of oligosaccharide assembly, tuning the protecting groups on the respective acceptor and donors was very essential, led to the design of new set of acceptors, donors and the whole glycosylation strategy.

### 2.3. Total Synthesis of PS A1 repeating unit with alternating charges on adjacent monosaccharides:

After the unsuccessful attempt towards the synthesis of PS A1 repeating unit, individual monosaccharide building blocks were designed to be more reactive and surpass the previous problems encountered in the total synthesis. In contrast to the initial attempt of introducing the amine functionality later, 2-azido-2,4,6-trideoxy-D-AAT was synthesized and used as a donor to obtain the challenging α(1,4) linkage between D-AAT and D-Gal residue.[33] Retrosynthetic analysis is shown in the Scheme 2. Completely deprotected PS A1 **1** was obtained through a one-pot, three-step global deprotection strategy of protected tetrasaccharide **2**, obtained through a [3+1] glycosylation strategy of trisaccharide acceptor **15** and pyruvate D-Gal*p* donor **16**. The orthogonally protected tetrasaccharide **2** was protected with Fmoc and *p*-methoxy phenol protecting groups at the non-reducing and reducing ends respectively for the synthesis of PS A1 fragments of higher molecular weight. The trisaccharide acceptor **15** was obtained using a [2+1] glycosylation of disaccharide acceptor **17** and D-AAT donor **18**, followed by *p*-TsOH mediated

deacetylation under mild acidic condition. The disaccharide acceptor **17** was synthesized from D-Gal*f* donor **19** and D-Gal*p* acceptor **20** using a [1+1] glycosylation strategy (Scheme 2). The choice of thio *vs* trichloroacetimidate donors used in the glycosylation reactions was based solely on the efficient reaction outcome.[33]

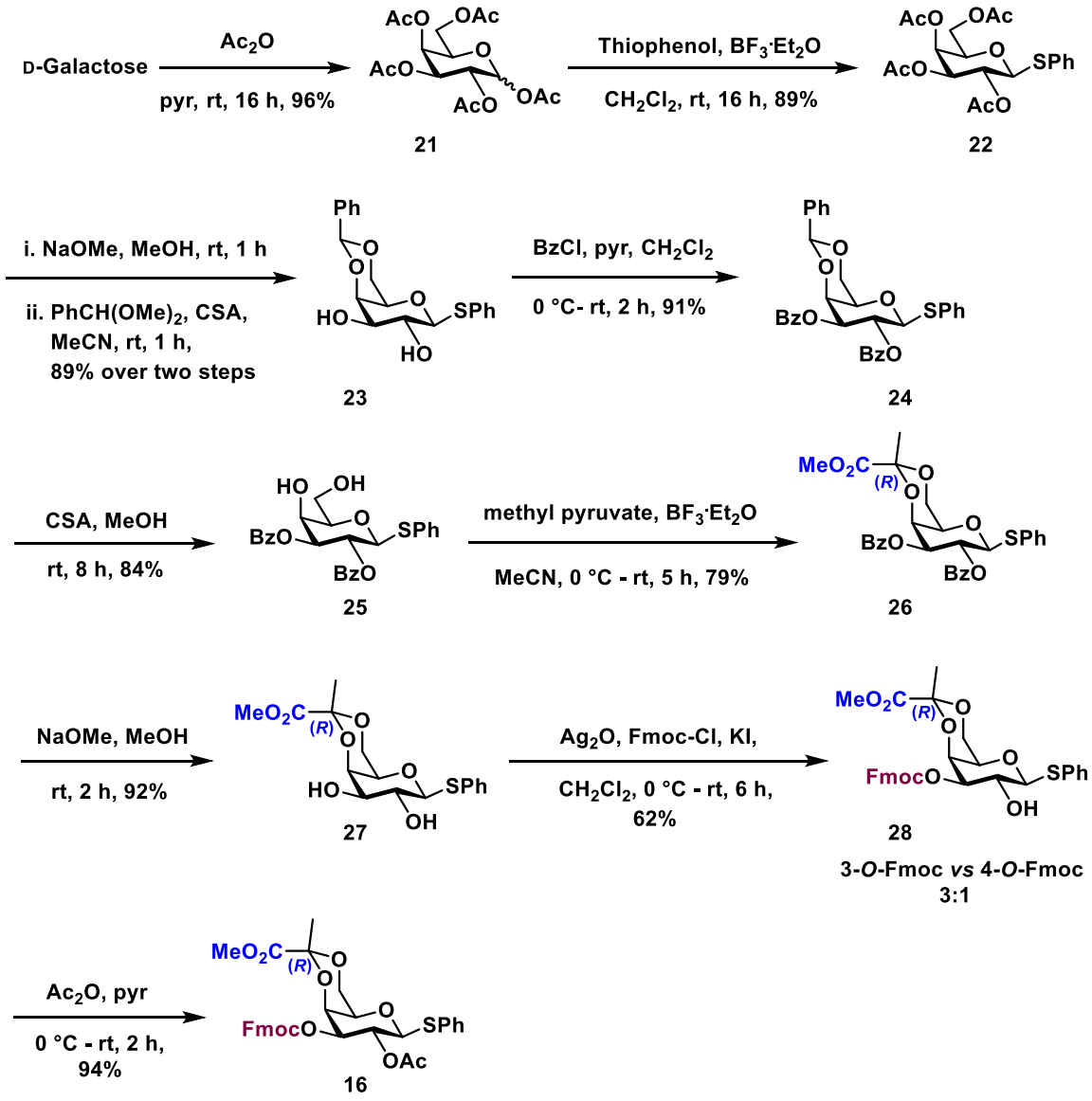



**Scheme 2:** Retrosynthetic analysis of tetrasaccharide repeating unit of PS A1

### 2.3.1. Synthesis of monosaccharide building blocks:

Synthesis of zwitterionic tetrasaccharide repeating unit of PS A1 **1** with alternating charges on adjacent monosaccharide residues began with the synthesis of monosaccharide building blocks pyruvate D-Gal*p* thiophenyl donor **16**,[126] rare residue 2-azido-4-trichloroethoxycarbamate-2,4,6-trideoxy-D-AAT trichloroacetimidate donor **18**,[33] D-Gal*f* trichloroacetimidate donor **19**,[114] *p*-methoxy phenyl-*O*-4,6-benzylidene-D-Gal$N_3$ acceptor **20**.[127]

**Synthesis of orthogonally protected pyruvate D-Galp donor 16:**



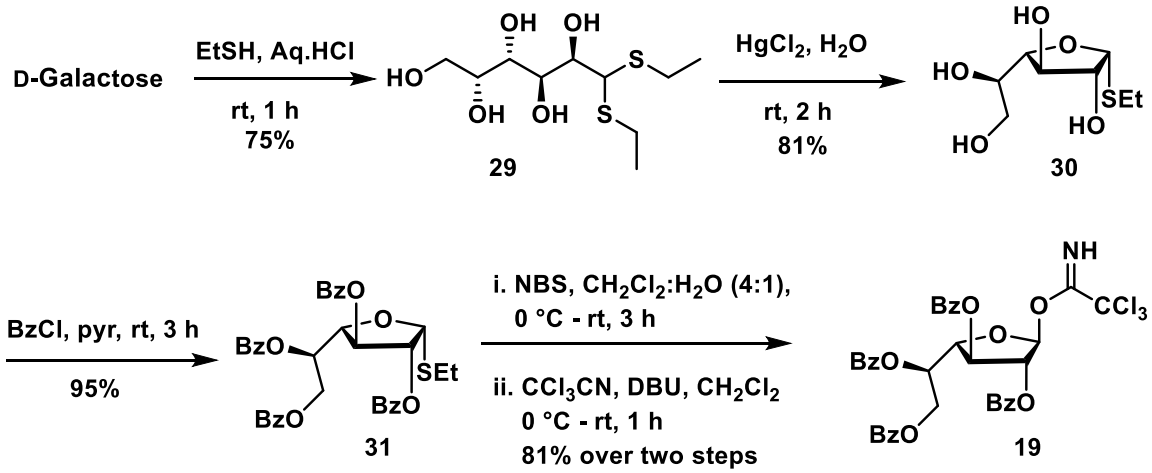

**Scheme 3:** Synthesis of orthogonally protected pyruvate D-Gal*p* donor **16**

Orthogonally protected pyruvate D-Gal*p* donor **16** synthesis (Scheme 3) began with peracetylation of D-galactose, followed by the installation of the thiophenyl group at the reducing end using $BF_3{\cdot}Et_2O$ to obtain the desired β-thiophenyl-2,3,4,6-OAc-D-Gal*p* **22** in 85% yield over two steps, encashing the anchimeric assistance of the 2-OAc ester

functionality (Scheme 3). The compound **22** was then subjected to Zemplén deprotection conditions to obtain the tetraol. The tetraol was then regio-selectively protected with benzylidene protecting group using benzylidene dimethyl acetal under acidic conditions to afford the desired 4,6-*O*-benzylidene protected β-thiophenyl D-Gal*p* compound **23** in 89% yield over two steps. The compound **23** was then protected with BzCl in pyridine at 0 ˚C to obtain the desired β-thiophenyl-2,3-di-OBz-4,6-benzylidene protected D-Gal*p* **24** in 91% yield.[126]

The benzylidene protecting group was deprotected under acidic conditions using camphorsulfonic acid (CSA) in methanol (MeOH) at room temperature to obtain compound **25** in 84% yield and followed by the pyruvate protection using methyl pyruvate and $BF_3{\cdot}Et_2O$ in acetonitrile to obtain the thio donor **26** in 79% yield.[126] The pyruvate protected compound **26** can be used directly as a donor in the glycosylation reaction, in order to access the orthogonally protected tetrasaccharide **2** for further repeating unit elongation of the tetrasaccharide, compound **26** was converted to orthogonally protected pyruvate D-Gal*p* thio donor **16** in 3 steps. Compound **26** was subjected to Zemplén deprotection to afford the desired 2,3-diol **27** in 92% yield. Silver (I) oxide mediated regioselective protection of C3-OH using FmocCl in dichloromethane (DCM) led to the formation of C3-Fmoc protected pyruvate galactose **28** in 62% yield and with a 3:1 ratio of C3 *vs* C2 Fmoc products separated by column chromatography.[128] Finally, the compound **28** was subjected to acetylation in pyridine at 0 ˚C to afford the desired orthogonally protected pyruvate D-Gal*p* thio donor **16** in 94% yield.

**Synthesis of D-Gal*f* trichloroacetimidate donor 19**:

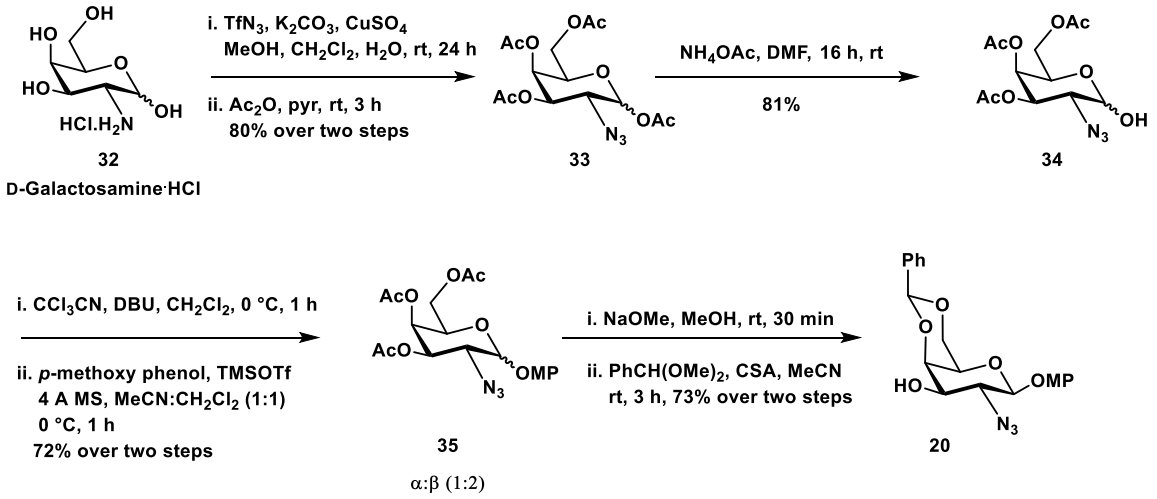



**Scheme 4:** Synthesis of trichloroacetimidate donor of D-Gal*f* **19**

D-galactose was converted to di thioacetal **29** using aq. HCl and ethanethiol at room temperature in 75% yield, followed by cyclization mediated by mercuric chloride ($HgCl_2$) in water at room temperature to afford the desired α-SEt-D-Gal*f* donor **30** in 81% yield. The compound **30** was then treated with BzCl in pyridine to obtain the desired per benzoylated product **31** in 95% yield. The α-SEt D-Gal*f* donor was then converted to the more reactive trichloroacetimidate donor **19** in two steps, first by the hydrolysis of the thioether using *N*-bromo succinimide (NBS) in dichloromethane and $H_2O$ to afford the hemiacetal and treated with trichloroacetonitrile in the presence of cat. DBU to obtain the desired trichloroacetimidate D-Gal*f* donor **19** in 81% yield over two steps (Scheme 4).

**Synthesis of β-p-methoxy phenyl-2-azido-4,6-O-benzylidine-D-Galp acceptor 20:**

The synthesis of the acceptor **20** (Scheme 5) started with diazotransfer reaction[129] of the D-galactosamine·HCl **32** using triflic azide ($TfN_3$), cat. $CuSO_4$ in a mixture of methanol, dichloromethane and water solvent system at room temperature over a period of 24 h, followed by peracetylation with $Ac_2O$ to obtain the desired azido compound **33** in 80% yield.



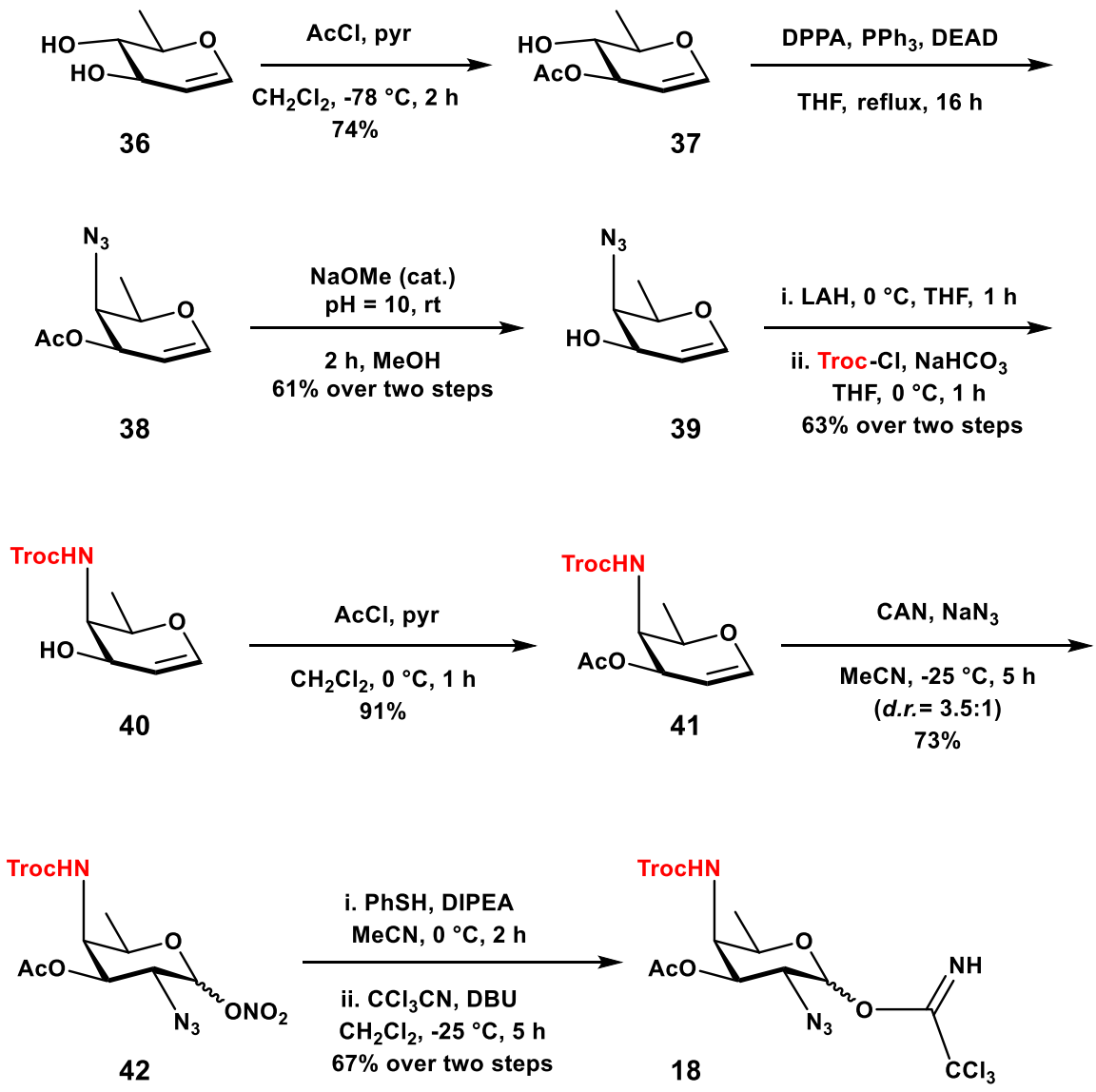

**Scheme 5:** Synthesis of D-Gal$N_3$ acceptor **20**

Regioselective anomeric deacetylation mediated by ammonium acetate ($NH_4OAc$) led to the formation of the hemiacetal **34** in 81% yield. Treatment of the hemiacetal with trichloroacetonitrile with cat. DBU in dichloromethane afforded the trichloroacetimidate donor, followed by the glycosylation of TCA donor with *p*-methoxy phenol using cat. TMSOTf led to the formation of the desired MP protected azido compound **35** ($\alpha:\beta$ = 1:2) in 72% yield over two steps. The compound **35** was subjected to Zemplén deprotection to obtain the triol intermediate which was subjected to regioselective benzylidene protection using benzylidene dimethyl acetal in acetonitrile to afford the desired 4,6-*O*-benzylidene protected Gal$N_3$ acceptor **20** in 73% yield over two steps.[127]

**Synthesis of the rare D-AAT trichloroacetimidate donor 18:**

Synthesis of the rare sugar residue D-AAT trichloroacetimidate donor **18** (Scheme 6) started from the highly regioselective protection of C3-OH of the D-rhamnal **36** using AcCl, pyridine at -78 ˚C in dichloromethane to afford exclusively 3-OAc protected D-rhamnal **37** in 74% yield. The 3-OAc protected D-rhamnal was subjected to Bose-

Mitsunobu reaction[130] to afford the desired azido compound **38**, followed by the deprotection of acetate protecting group under Zemplén conditions to afford the desired C3-alcohol **39** in 61% yield over two steps. Direct reduction of azide with the C3-OAc protected compound **38** led to the formation of acetyl group migration onto amine to afford the undesired acetamido compound under various conditions like ethane dithiol mediated reduction, LAH and Staudinger reaction. With the desired azido alcohol **39** in hand, compound **39** was subjected to afore mentioned azide to amine reduction protocols and LAH mediated reduction worked best affording the desired amino alcohol. The crude amino alcohol was then subjected to 2,2,2-trichloroethoxy carbonyl chloride (TrocCl), sodium bicarbonate ($NaHCO_3$) in tetrahydrofuran (THF) to afford the desired carbamate **40** in 63% yield over two steps. The carbamate **40** was protected with AcCl using pyridine as a base afforded the desired compound **41** in 91% yield. Azidonitration[131] of compound **41** using ceric ammonium nitrate (CAN) and sodium azide ($NaN_3$) in acetonitrile (MeCN) at -25 ˚C afforded the desired compound **42** in 73% yield with 3.5:1 ratio of the C2 epimers (galacto:talo = 3.5:1) calculated as by $^1H$ NMR. Compound **42** was then converted to hemiacetal using thiophenol, followed by treating the hemiacetal with $CCl_3CN$ and cat. DBU to afford the desired trichloroacetimidate D-AAT donor **18** in 67% yield over two steps.




**Scheme 6:** Synthesis of the rare D-AAT trichloroacetimidate donor **18**

### 2.3.2. Assembly of monosaccharide building blocks: PS A1 repeating unit

With the required building blocks in hand, the assembly of the monosaccharide building blocks was started with [1+1] glycosylation of trichloroacetimidate donor of D-Gal*f* **19** and *p*-methoxy phenyl-4,6-*O*-benzylidene-D-Gal*p* acceptor **20**. At first the α-MP protected galactose azido acceptor **20a** (Figure 13) was used in the [1+1] glycosylation, very surprisingly aglycone transfer of the methoxy phenyl group was observed.[132] The

reason for the observed aglycone transfer was due the highly electron rich nature of the α-glycosidic linkage in the acceptor, because of the anomeric effect of the ring oxygen and also electron donating ability of the *p*-methoxy group, and the second driving force for the aglycone transfer is due to the relative stability of the oxocarbenium ions generated from the acceptor **20i** (after aglycone transfer) *vs* donor **19i** (Figure 13).

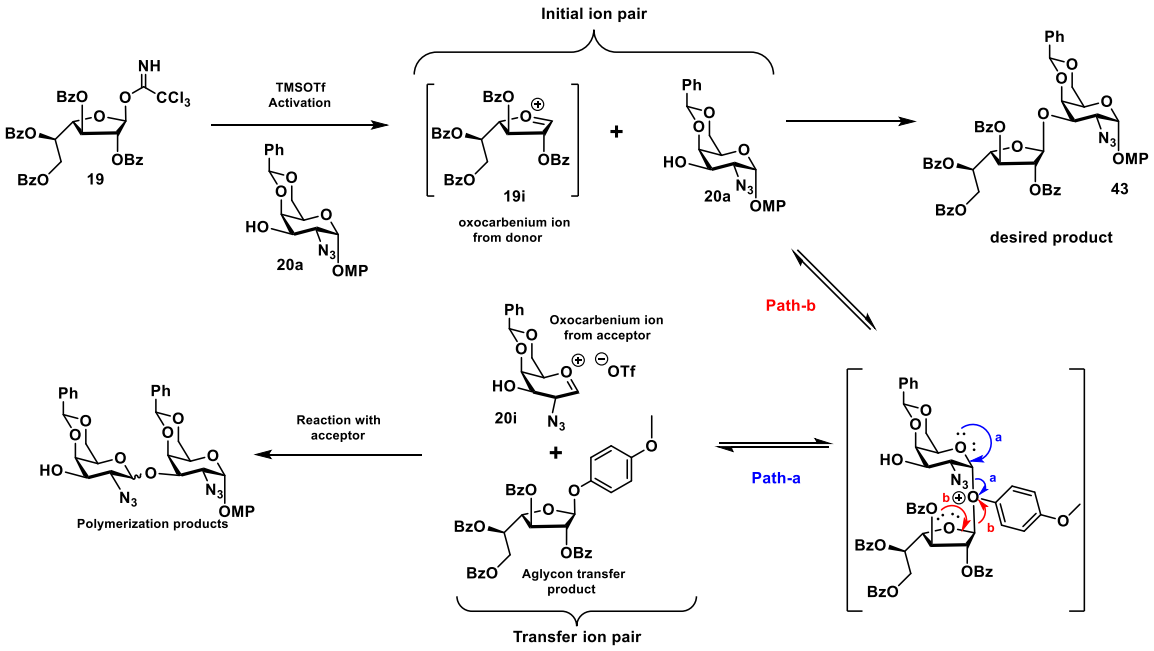
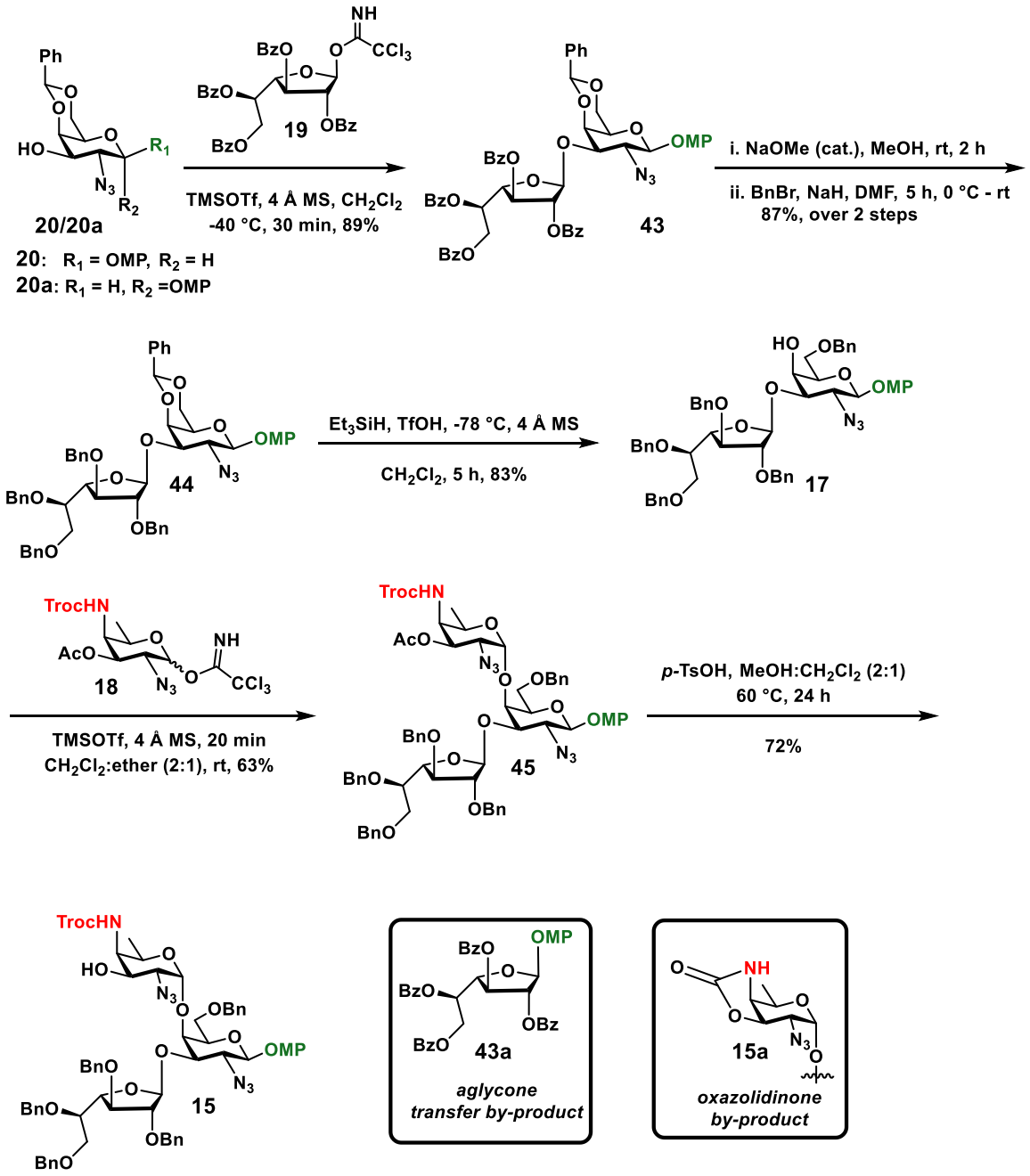



**Figure 2 - 2:** Plausible mechanism for aglycone transfer

In detail, the plausible mechanism was shown (Figure 13), activation of the TCA donor **19** by TMSOTf will lead to the formation of an initial ion pair **19i**/**20a**, consisting of oxocarbenium ion generated from the TCA donor **19i** and acceptor **20a**. In case, the C3-OH of the acceptor reacts with the oxocarbenium ion from the donor **19i**, it will lead to the expected product **43** (path-b, Figure 13). Initial ion pair can take a different pathway due to the competing, electron rich α-glycosidic linkage of the acceptor, leading to the aglycone transfer (path-a, Figure 13) and hence leading to the formation of the transfer ion pair. The

newly formed oxocarbenium ion **20i** due to the aglycone transfer can react with the acceptor **20a** giving rise to polymerization by-products. Hence, the electron rich nature of the α-glycosidic linkage and the relative stabilities of the oxocarbenium ions from the donor **19i** and acceptor **20i** might be the driving force behind the very rare *O*-glycosidic aglycone transfer.

The above aglycone transfer was circumvented using the β-isomer of the acceptor **20**, as minute changes to the glycosylation reaction parameters can lead to or avoid aglycone transfer.[132] Using [1+1] glycosylation strategy, the TCA donor of D-Gal*f* **19** was coupled with β-*p*-methoxy phenol-4,6-*O*-benzylidene-D-Gal*p* acceptor **20** using Schmidt's glycosylation strategy at -40 ˚C to obtain the desired disaccharide **43** in 89% yield.[122] The disaccharide **43** was then subjected to Zemplén conditions to deprotect the benzoate esters on the D-Gal*f* residue and then the crude tetraol was then benzylated with benzyl bromide (BnBr), sodium hydride (NaH) in *N*, *N*-dimethyl formamide (DMF) at 0 ˚C to afford the desired benzyl protected disaccharide **44** in 87% yield over two steps (Scheme 7). The compound **44** was then subjected to regioselective benzylidene ring opening[133] using triethyl silane ($Et_3SiH$) and triflic acid (TfOH) at -78 ˚C in DCM to afford the desired disaccharide acceptor **17** in 83% yield. The reason for switching from benzoyl to benzyl protecting groups on D-Gal*f* residue is due to the very well-known low reactivity of the C4-OH on galactose (Scheme 7).[110, 114]




**Scheme 7:** Synthesis of the trisaccharide acceptor **15**

With the disaccharide acceptor **17** in hand, the challenging 1,2-*cis* or α(1,4) linkage between the rare sugar D-AAT donor **18** and the C4-OH of the disaccharide acceptor **17** was screened under various glycosylation protocols.[33] Careful tuning of various glycosylation reaction parameters like temperature, mol % of the catalyst, various Bronsted acids, concentration of the reaction mixture, proper ratio of the solvent mixture and equivalents of the donor/acceptor afforded the desired trisaccharide **45** in 63% yield with complete α-selectivity (Scheme 7).[33] The reason for the high α-selectivity is believed due to the remote participation of the NHTroc protecting group in the oxocarbenium ion intermediate (Figure 14).[134]

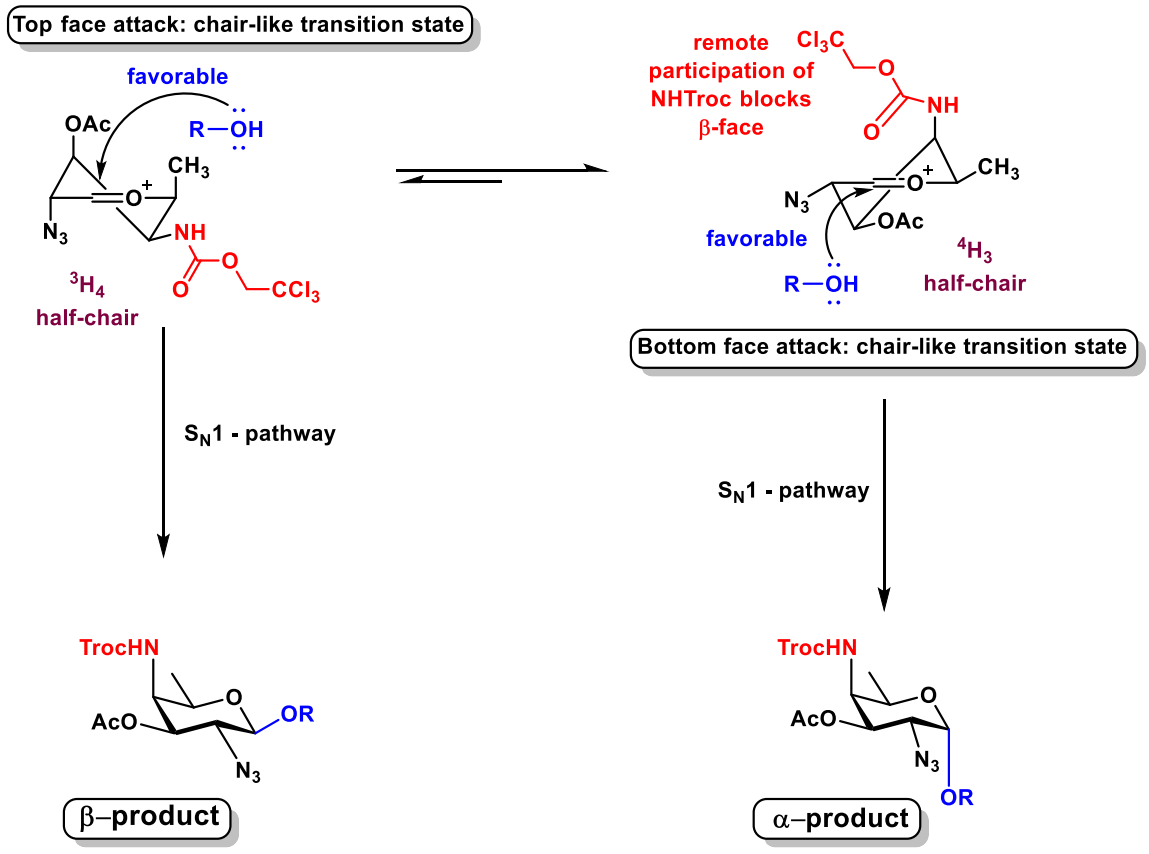
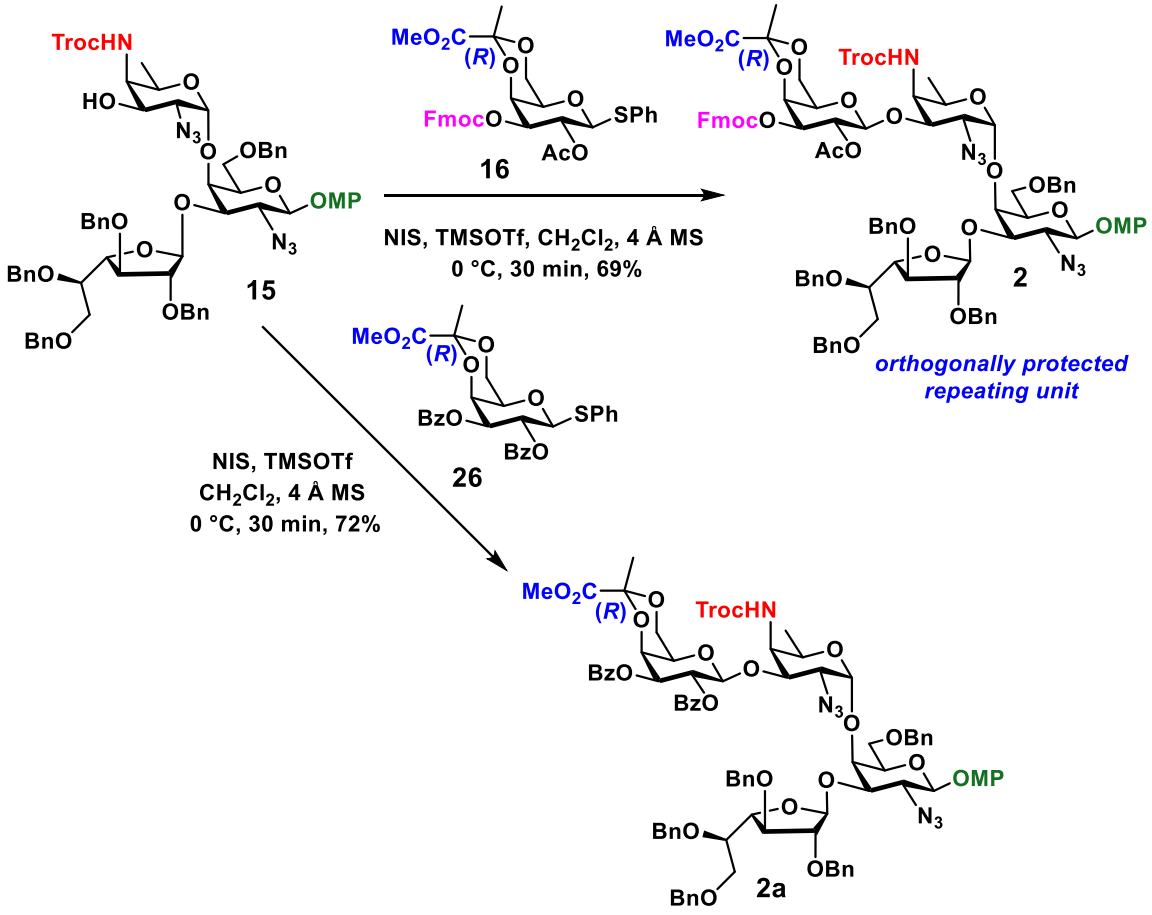



**Figure 2 - 3:** Plausible mechanism for remote participation mediated high α-selectivity

The oxocarbenium ion formed after the activation of TCA donor by TMSOTf can exist in two half-chair conformations, the less stable $^{3}H_{4}$ conformation *vs* more stable $^{4}H_{3}$ conformation. In the $^{3}H_{4}$ conformation, the top-face attack of the nucleophile/acceptor will go through a chair-like transition state affording the 1,2-*trans* or β-glycoside, whereas the bottom face attack of the nucleophile will lead to the formation of 1,2-*cis* or α-glycoside through a highly strained boat-like transition state. As the $^{3}H_{4}$ conformation is very unstable due to the presence of the substituents in the axial position and raising the overall energy of the intermediate, the equilibrium between the $^{3}H_{4}$ *vs* $^{4}H_{3}$ conformations is shifted more towards the stable $^{4}H_{3}$ conformation. In the $^{4}H_{3}$ conformation, the top face attack of the nucleophile/acceptor will lead to the formation of 1,2-*trans* or β-glycoside through a highly unfavorable boat-like transition state and top face attack is sterically less feasible because of the remote participation of NHTroc protecting group blocking the top face attack, analogous to the very common C2-ester anchimeric assistance mediated formation of the 1,2-*trans* glycosides. In conclusion, the exclusive α-selectivity observed in the [2+1] glycosylation between the disaccharide acceptor **17** and D-AAT donor **18** might be due to the remote participation of the NHTroc protecting group, blocking the top face attack and leading to the bottom face attack of nucleophile through a stable chair-like transition state resulting in the formation of 1,2-*cis* or α-glycoside exclusively. The conformation of the oxocarbenium ion is mainly dependent on the temperature of the glycosylation reaction and stereoselective outcome is known to be dependent on various factors like solvent, temperature, nature of the acceptor and others. The deacetylation of the C3-OAc protected trisaccharide **45** was attempted under various reaction conditions like Zemplén, Guanidinium nitrate ($GHNO_3$)[135] mediated deacetylation in the presence of NHTroc and

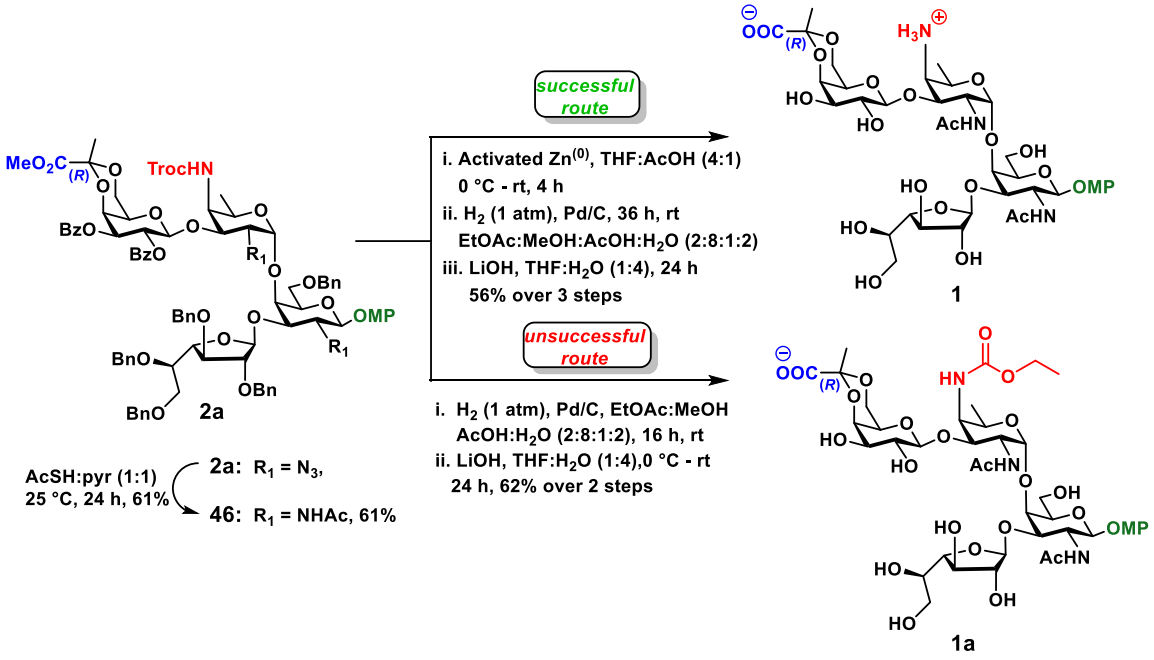
triethylamine (pH = 8) to obtain the desired trisaccharide acceptor **15**. None of the above-mentioned reaction conditions, even with the very mild $GHNO_3$ mediated deacetylation afforded the desired trisaccharide acceptor **15**, whereas the undesired oxazolidinone by-product **15a** was isolated. The reason for the observed cyclization is due to the *cis*-nature of the C3-OH and NHTroc groups leading to the oxazolidinone ring formation. After the failure in basic reaction conditions, deacetylation was attempted in acidic conditions using *p*-TsOH in a 2:1 mixture of methanol and DCM, the desired trisaccharide acceptor **15** was obtained in 72% yield (Scheme 7).[136]




**Scheme 8:** Synthesis of orthogonally protected tetrasaccharide **2**

With the trisaccharide acceptor **15** and two different pyruvate D-Gal*p* donors in hand, the [3+1] glycosylation was attempted (Scheme 8). The standard thioglycoside activation condition using *N*-iodo succinimide (NIS) and TMSOTf was used to couple the trisaccharide acceptor **15** and orthogonally protected pyruvate D-Gal*p* **16** to obtain the desired orthogonally protected tetrasaccharide **2** in 69% yield. Similar [3+1] glycosylation with the trisaccharide acceptor **15** using the other pyruvate D-Gal*p* donor **26** led to the formation of the protected PS A1 tetrasaccharide **2a** in 72% yield (Scheme 8).

With the protected PS A1 tetrasaccharide **2a** in hand, the global deprotection was attempted to obtain the completely deprotected PS A1 tetrasaccharide repeating unit (Scheme 9). Azido groups in the protected PS A1 were converted to acetamido groups in one-pot, one-step using AcSH and pyridine to obtain the desired diacetamido tetrasaccharide **46** in 61% yield.[123]




**Scheme 9:** Synthesis of zwitterionic repeating unit of PS A1 **1**

Initial attempt was to deprotect the benzyl ethers followed by the saponification of benzoate esters, in the same step, the base labile NHTroc protecting group will be converted to amine through the unstable carbamate. Surprisingly, the debenzylation with Pd/C in the presence of solvent system buffered with AcOH led to the loss of three chlorine atoms in the NHTroc functionality to afford the undesired ethyl carbamate.[137] The respective ethyl carbamate was subjected to saponification using LiOH, the benzoate esters were cleaved but the ethyl carbamate was still intact and required heating to afford the undesired compound **1a**. As the PS A1 tetrasaccharide has NHAc functional groups which are not stable to strong basic conditions under heating, the harsh conditions to remove ethyl carbamate was not attempted. Instead, a one-pot, three-step deprotection strategy was attempted to obtain the desired compound. First, the NHTroc group was deprotected using $Zn^{(0)}$ in AcOH/THF to obtain the respective amine,[101] followed by the hydrogenation of the benzyl groups and finally the benzoate esters were deprotected using LiOH to obtain the zwitterionic PS A1 tetrasaccharide **1** in 56% yield over three steps.[33]

## 2.4. Conclusion:

Synthesis of zwitterionic tetrasaccharide repeating unit of polysaccharide A1 (PS A1) **1** was accomplished using a linear glycosylation strategy. Few challenging steps like avoiding the aglycone transfer, α(1,4) glycosidic linkage between the rare sugar D-AAT and low reactive C4-OH of D-Gal*p* residue in the disaccharide acceptor, acid-mediated deacetylation and final global deprotection sequence have been well-thought and executed. The final *p*-methoxy phenyl protected PS A1 tetrasaccharide **1** and the corresponding hemiacetal will be tested *in vivo* in mice and binding studies with MHC II (HLA-DR2) will be studied using ITC experiments.

## 2.5. Experimental section:

### General Information: Experiments, Materials and Equipment

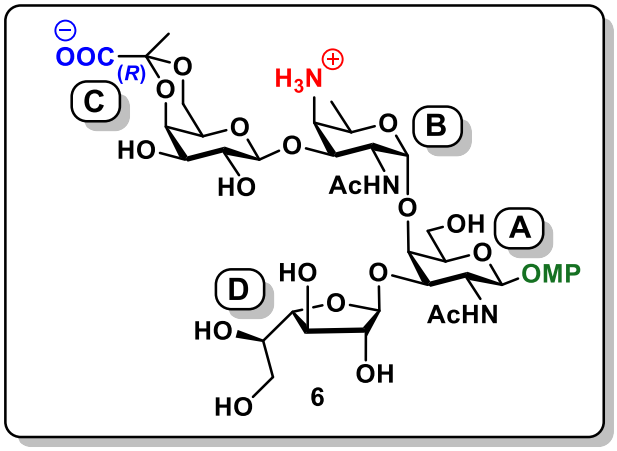
All reactions were carried out using dry solvents, oven dried glassware and under inert atmosphere (Argon balloon) unless otherwise noted. All chemicals/reagents and solvents were obtained from commercial sources (Sigma, Oakwood, Alfa and AK Scientific) and used without any further purification. Solvents for workup and silica gel flash chromatography were obtained from Sigma. Molecular sieves (4 Å) were dried/activated overnight at 140 ºC under vacuum with an inert gas connection and further activated using a heat gun under high vacuum for 10 min and cooled to room temperature under high vacuum. Reaction transformations were monitored using Silicycle® silica gel plates coated with silica gel (catalog number: TLG-R10014B-323, 0.25 mm thickness), visualized under UV chamber and stained using 5% con. $H_2SO_4$ in methanol/ethanol or *p*-anisaldehyde staining solution (135 mL ethanol, 5 mL con. $H_2SO_4$, 1.5 mL glacial AcOH and 3.7 mL *p*-anisaldehyde). Siliaflash® P60 (catalog number: R12030B, 230-400 mesh) was used with a 75-100:1 weight ratio to the crude compound for purification. All NMR experiments ($^1$H, $^{13}$C, COSY, HSQC, DEPT135, 1D TOCSY and HMBC) were performed on a Bruker Avance III (600 MHz with cryoprobe and Z gradient). Chemical shifts were reported in δ ppm respective to the internal standard of the residual chloroform ($^1$H: 7.26 ppm, $^{13}$C: 77.16 ppm), methanol ($^1$H: 3.31 ppm, $^{13}$C: 49.1 ppm) or $D_2O$ ($^1$H: 4.79 ppm). Proton NMR data are reported as follows: chemical shift (δ ppm), multiplicity (s: singlet, d: doublet, dd: doublet of doublet, ddd: doublet of doublet of doublets, t: triplet and m: multiplet), coupling constant (*J* in Hz), integration and respective assigned proton(s). $^{13}$C NMR data are reported as follows: chemical shift (δ ppm) and respective assigned carbon.

Electron spray ionization (ESI) was used for low resolution mass and HRMS time-of-flight (TOF) techniques were used to obtain low- and high-resolution mass spectra (Water synapt HDMS/nano ESI-MS). Optical rotation data was collected on Rudolph Analytical (Autopol IV with Temp Trol). Reaction yields, for all the reactions carried out, refer to chromatographically and spectroscopically pure compound, unless otherwise noted.

**Abbreviations:**

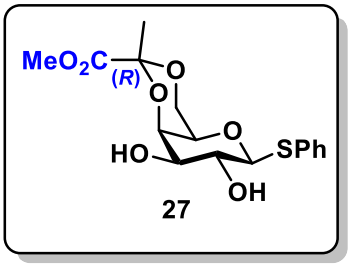
THF = tetrahydrofuran, DMF = *N*, *N*-dimethyl formamide, AcOH = acetic acid, EtOAc = ethyl acetate, *p*-TsCl = 4-toluenesulfonyl chloride, $NaHCO_3$ = sodium bicarbonate, $Na_2SO_4$ = sodium sulfate, LAH = lithium aluminum hydride, $NH_4Cl$ = ammonium chloride, $PPh_3$ = triphenylphospine, DEAD = diethyl azodicarboxylate, NaH = sodium hydride, anhyd = anhydrous, $NaN_3$ = sodium azide, DIPEA = *N*, *N*-diisopropylethylamine, DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, NBS = *N*-bromosuccinimide, $Na_2S_2O_3$ = sodium thiosulfate, $BF_3{\cdot}Et_2O$ = boron trifluoride diethyl etherate, TMSOTf = trimethylsilyl trifluoromethanesulfonate, NaOMe = sodium methoxide, TfOH = triflic acid, *p*-TsOH = *p*-toluenesulfonic acid, NIS = *N*-bromosuccinimide, rt = room temperature.

Proton assignment based on the following figure:



**Experimental Procedures:**

**4,6-*O*-[1-(*R*)-(methoxycarbonyl)-ethylidene]-1-thio-β-D-galactopyranoside (27):**




27

Compound **26** (0.5 g, 0.89 mmol, 1.0 equiv) was dissolved in methanol (10 mL) and then freshly prepared 1 M NaOMe (1 mL, pH = 9) was added and the reaction mixture was stirred for 2 h at room temperature. Amberlite resin ($H^+$) was added to the reaction mixture and stirred until the pH = 6 (pH paper), then the reaction mixture was filtered over a cotton plug to remove Amberlite® resin. The solvent was removed under reduced pressure to obtain the unpurified compound. Purification of the crude compound using silica gel column chromatography (gradient method-hexane/EtOAc, 3:2) afforded the desired compound **27** (0.29 g, 92% yield) as a white solid.

Data for compd **27**:

$^1$H NMR: (600 MHz, $CDCl_3$)

δ 7.65-7.63 (m, 2H, Ar-*H*), 7.32-7.31 (m, 3H, Ar-*H*), 4.48 (d, *J* = 9.1 Hz, 1H, $1_C$), 4.14-4.11 (m, 2H, $4_C$, $6_C$), 3.98 (d, *J* = 12.7 Hz, 1H, $6_C$), 3.81 (s, 3H, OC$H_3$), 3.67-3.62 (m, 2H, $2_C$, $3_C$), 3.45 (s, 1H, $5_C$), 2.63 (d, *J* = 7.6 Hz, 1H, O*H*), 2.57 (s, 1H, O*H*), 1.53 (s, 3H, pyruvate C$H_3$)

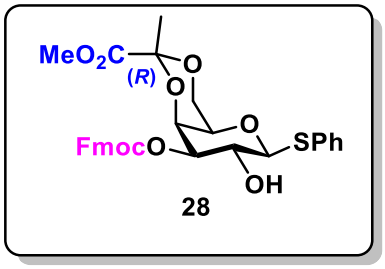
$^{13}$C NMR: (150 MHz, $CDCl_3$)

δ 170.2 (*C*$O_2$Me), 133.5 (Ar-*C*), 131.2 (Ar-*C*), 129.0 (Ar-*C*), 128.3 (Ar-*C*), 98.6 (pyruvate quaternary *C*), 87.4 ($1_C$), 73.7 ($3_C$), 71.1 ($4_C$), 69.2 ($5_C$), 68.8 ($2_C$), 65.5 ($6_C$), 52.9 (O*C*$H_3$), 25.8 (pyruvate *C*$H_3$)

ESI-MS: Exact mass calcd. for $C_{16}H_{20}O_7S^+$ $[M+Na]^+$: 379.1, found 378.8

TLC: $R_f$ = 0.2 (hexane/EtOAc, 3:2)

**Phenyl 3-*O*-fluorenylmethyloxycarbonyl-4,6-*O*-[1-(*R*)-(methoxycarbonyl)-ethylidene]-1-thio-β-D-galactopyranoside (28):**




28

Compound **27** (0.28 g, 0.79 mmol, 1.0 equiv) was dissolved in anhyd. $CH_2Cl_2$ (12 mL), silver (I) oxide (0.29 g, 1.26 mmol, 1.6 equiv) and KI (0.026 g, 0.16 mmol, 0.2 equiv) were added at 0 ºC. After a short period of time (5 min) Fmoc-Cl (0.23 g, 0.87 mmol, 1.1 equiv) was added drop-wise at 0 ºC and the reaction was allowed to stir at room temperature for 6 h. After TLC showed complete reaction of the starting material, reaction mixture was quenched with methanol (1 mL), diluted with $CH_2Cl_2$ (30 mL), filtered over Celite® bed and the filtrate was washed with aq $NaHCO_3$ (1 x 25 mL). The organic layer was separated, dried over $Na_2SO_4$ and removed under vacuum to afford the crude compound. Purification of the crude compound, using silica gel chromatography (gradient method-hexane/EtOAc, 3:1), afforded the desired compound **28** (0.282 g, 62% yield) as a white solid.

Data for compd **28**:

$^{1}$H NMR: (600 MHz, $CDCl_3$)

δ 7.77 (d, *J* = 7.6 Hz, 2H, Ar-*H*), 7.69-7.67 (m, 2H, Ar-*H*), 7.64-7.61 (m, 2H, Ar-*H*), 7.41 (t, *J* = 7.5 Hz, 2H, Ar-*H*), 7.35-7.34 (m, 3H, Ar-*H*), 7.31-7.28 (m, 2H, Ar-*H*), 4.68 (dd, *J* = 9.7, 3.5 Hz, 1H, $3_C$), 4.56 (d, *J* = 9.5 Hz, 1H, $1_C$), 4.49-4.44 (m, 2H, C*H*H of Fmoc, $4_C$), 4.35-4.32 (m, 2H, CH*H* of Fmoc, C*H* of Fmoc), 4.15 (d, *J* = 12.6, 1H, $6_C$), 4.00-3.94 (m, 2H, $6_C$, $2_C$), 3.58 (s, 3H, OC$H_3$), 3.49 (d, *J* = 1.2 Hz, 1H, $5_C$), 2.49 (s, 1H, O*H*), 1.54 (s, 3H, pyruvate C$H_3$)

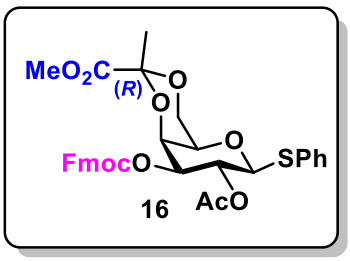
$^{13}$C NMR: (150 MHz, $CDCl_3$)

δ 170.1 (*C*$O_2$Me), 154.5 ($CH_2OCO$-Fmoc), 143.4 (Ar-*C*), 141.4 (Ar-*C*), 141.3 (Ar-*C*), 133.8 (Ar-*C*), 130.5 (Ar-*C*), 129.1 (Ar-*C*), 128.6 (Ar-*C*), 128.0 (Ar-*C*), 127.3 (Ar-*C*), 127.3 (Ar-*C*), 125.5 (Ar-*C*), 125.4 (Ar-*C*), 120.1 (Ar-*C*), 98.6 (pyruvate quaternary *C*), 87.5 ($1_C$), 78.2 ($3_C$), 70.5 (*C*$H_2$-Fmoc), 69.0 ($5_C$), 68.9 ($4_C$), 65.6 ($2_C$), 65.5 ($6_C$), 52.6 (O*C*$H_3$), 46.6 (*C*H-Fmoc), 25.7 (pyruvate *C*$H_3$)

ESI-MS: Exact mass calcd. for $C_{31}H_{30}O_9S^+$ $[M+Na]^+$: 601.2, found 601.0

TLC: $R_f$ = 0.4 (hexane/EtOAc, 3:1)

**Phenyl 2-*O*-acetyl-3-*O*-fluorenylmethyloxycarbonyl-4,6-*O*-[1-(*R*)-(methoxycarbonyl)-ethylidene]-1-thio-β-D-galactopyranoside (16):**



Compound **28** (0.26 g, 0.45 mmol, 1.0 equiv) was dissolved in pyridine (3 mL) and then acetic anhydride (1 mL) was added at 0 ºC. The reaction was allowed to stir at room temperature for 2 h. After TLC showed complete reaction of the starting material, solvents were removed under reduced pressure and ultimately co-distilled with toluene (2 x15 mL) to obtain the unpurified compound. Purification of the crude compound, using silica gel chromatography (gradient method-hexane/EtOAc, 3:1), afforded the desired compound **16** (0.262 g, 94% yield) as a colorless gummy liquid.

Data for compd **16**:

$^{1}$H NMR: (600 MHz, $CDCl_3$)

δ 7.76 (d, *J* = 7.6 Hz, 2H, Ar-*H*), 7.61-7.59 (m, 4H, Ar-*H*), 7.42-7.39 (t, *J* = 7.4 Hz, 2H, Ar-*H*), 7.34-7.29 (m, 5H, Ar-*H*), 5.42 (t, *J* = 9.9 Hz, 1H, $2_C$), 4.77 (dd, *J* = 9.9, 3.5 Hz, 1H, $3_C$), 4.68 (d, *J* = 9.9 Hz, 1H, $1_C$), 4.48 (d, *J* = 3.5 Hz, 1H, $4_C$), 4.44-4.41 (m, 1H, C*H*H of Fmoc), 4.32-4.29 (m, 2H, C*H* of Fmoc, CH*H* of Fmoc), 4.15 (d, *J* = 12.9 Hz, 1H, $6_C$), 3.96 (d, *J* = 12.9 Hz, 1H, $6_C$), 3.59 (s, 3H, OC$H_3$), 3.49 (d, *J* = 1.1 Hz, 1H, $5_C$), 2.11 (s, 3H, C$H_3$CO), 1.54 (s, 3H, pyruvate C$H_3$)

$^{13}$C NMR: (150 MHz, $CDCl_3$)

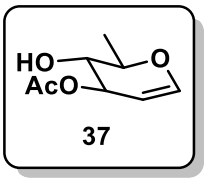
δ 170.1 (*C*$O_2$Me), 169.3 (*C*O$CH_3$), 154.3 ($CH_2$O*C*(O) of Fmoc), 143.4 (Ar-*C*), 143.2 (Ar-*C*), 141.4 (Ar-*C*), 141.3 (Ar-*C*), 133.6 (Ar-*C*), 128.9 (Ar-*C*), 128.3 (Ar-*C*), 128.1 (Ar-*C*), 128.0 (Ar-*C*), 127.3 (Ar-*C*), 125.6 (Ar-*C*), 125.4 (Ar-*C*), 120.2 (Ar-*C*), 120.1 (Ar-*C*), 98.7 (pyruvate quaternary *C*), 85.5 ($1_C$), 76.6 ($3_C$), 70.6 (*C*$H_2$ of Fmoc), 68.8 ($5_C$), 68.7 ($4_C$), 66.5 ($2_C$),

65.3 ($6_C$), 52.6 ($OCH_3$), 46.5 (*C*H of Fmoc), 25.7 (pyruvate $CH_3$), 21.1 ($CH_3CO$)

ESI-MS: Exact mass calcd. for $C_{33}H_{32}O_{10}S^+$ $[M+Na]^+$: 643.2, found 643.0

TLC: $R_f$ = 0.5 (hexane/EtOAc, 3:1)

**3-*O*-acetyl-6-deoxy-D-rhamnal (37):**




37

Compound **36**[138] (12 g, 92.31 mmol, 1.0 equiv) was dissolved in anhyd. $CH_2Cl_2$ (400 mL) and then pyridine (22.3 mL, 276.93 mmol, 3.0 equiv) was added and the reaction mixture was cooled to –78 °C. Acetyl chloride (7.3 mL, 101.54 mmol, 1.1 equiv) was added slowly, using a dropping funnel, over a period of 30 min and the reaction was allowed to stir at -78 ˚C for 2 h. After TLC showed complete reaction of the starting material, methanol (5 mL) was added slowly, using a dropping funnel, to the reaction mixture. The reaction mixture was then washed with sat. aq $NaHCO_3$ (2 x 50 mL), followed by 5% v/v cold aq HCl (1 x 125 mL) and washed again with sat. aq $NaHCO_3$ (1 x 100 mL) to remove any traces of acid. The organic layer was separated, dried over $Na_2SO_4$, filtered and the solvent was removed under reduced pressure to obtain unpurified compound. Silica gel chromatography (gradient method-hexane/EtOAc, 6:1) of the crude compound afforded the desired compound **37** (11.8 g, 74% yield) as a colorless oil.

Data for compd **37**:

$^1$H NMR: (600 MHz, $CDCl_3$)

δ 6.43 (dd, $J$ = 6.1, 1.2 Hz, 1H, $1_B$), 5.25-5.20 (m, 1H, $3_B$), 4.68 (dd, $J$ = 6.1, 2.5 Hz, 1H, $2_B$), 3.93-3.88 (m, 1H, $5_B$), 3.62-3.59 (m, 1H, $4_B$), 3.23 (s, 1H, -O*H*), 2.12 (s, 3H, COC$H_3$), 1.40 (d, $J$ = 6.4 Hz, 3H, C$H_3$/$6_B$)

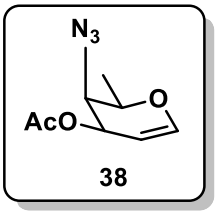
$^{13}$C NMR: (150 MHz, $CDCl_3$)

δ 173.1 (*C*O$CH_3$), 146.7 ($1_B$), 98.9 ($2_B$), 74.9 ($5_B$), 74.2 ($3_B$), 72.8 ($4_B$), 21.3 (CO*C*$H_3$), 17.2 (*C*$H_3$/$6_B$)

ESI-MS: Exact mass calcd. for $C_8H_{12}O_4^+$ $[M+Na]^+$: 195.1, found 195.0

TLC: $R_f$ = 0.35 (hexane/EtOAc, 6:1)

**3-*O*-acetyl-4-azido-4,6-dideoxy-D-galactal (38):**




To a solution of compound **37** (11.8 g, 68.61 mmol, 1.0 equiv) in anhyd. THF (200 mL), were added $PPh_3$ (21.6 g, 82.33 mmol, 1.2 equiv), DEAD (14.33 g, 82.33 mmol, 1.2 equiv) and diphenyl phosphoryl azide (30.9 g, 82.33 mmol, 1.2 equiv) respectively. The reaction mixture was then heated to reflux for 16 h. After TLC showed complete reaction of the starting material, the solvent was removed under reduced pressure, the residue was then diluted with EtOAc (200 mL), washed with water (1 x 100 mL) and finally organic layer was separated. The organic layer was dried over $Na_2SO_4$, filtered and the solvent was removed under reduced pressure to obtain unpurified compound. Purification of the crude material using silica gel chromatography (gradient method-hexane/EtOAc, 6:1) afforded the desired compound **38** as a colorless oil. ***Note:*** We were unable to separate compound

**38** from the by-product (UV-active), formed in this step, using silica gel chromatography. The by-product was separated out in the next deacetylation step.

Data for compd **38**:

$^{1}$H NMR: (600 MHz, $CDCl_3$)

δ 6.44 (dd, *J* = 6.3, 1.4 Hz, 1H, $1_B$), 5.60-5.59 (m, 1H, $3_B$), 4.70 (dt, *J* = 6.3, 1.7 Hz, 1H, $2_B$), 4.14 (q, *J* = 6.54 Hz, 1H, $5_B$), 3.86 (d, *J* = 4.92 Hz, 1H, $4_B$), 2.15 (s, 3H, COC*H*$_3$), 1.39 (d, *J* = 6.5 Hz, 3H, C*H*$_3$/$6_B$)

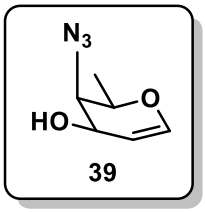
$^{13}$C NMR: (150 MHz, $CDCl_3$)

δ 170.8 (*C*O$CH_3$), 146.5 ($1_B$), 98.4 ($2_B$), 72.1 ($5_B$), 67.9 ($3_B$), 58.4 ($4_B$), 20.8 (CO*C*H$_3$), 17.6 (*C*H$_3$/$6_B$)

ESI-MS: Exact mass calcd. for $C_8H_{11}N_3O_3^+$ $[M+Na]^+$: 220.1, found 220.3

TLC: $R_f$ = 0.7 (hexane/EtOAc, 6:1)

**4-azido-4,6-dideoxy-D-galactal (39):**



39

To a solution of compound **38** in methanol (100 mL), was added freshly prepared 1 M sodium methoxide (10 mL, pH = ~10) and the reaction mixture was stirred for 2 h at room temperature. After TLC showed complete reaction of the starting material, the organic solvent was removed under reduced pressure. The residue was then subjected to purification using silica gel chromatography (gradient method-hexane/EtOAc, 6:1) and the desired compound **39** (6.5 g, 61% yield; over two steps) was obtained as a white solid.

Data for compd **39**:

$^{1}$H NMR: (600 MHz, $CDCl_3$)

δ 6.34 (dd, *J* = 6.2, 1.7 Hz, 1H, $1_B$), 4.68 (dt, *J* = 6.2, 1.8 Hz, 1H, $2_B$), 4.59 (m, 1H, $3_B$), 4.13 (q, *J* = 6.5 Hz, 1H, $5_B$), 3.65 (d, *J* = 5.2 Hz, 1H, $4_B$), 2.16 (d, *J* = 9.4 Hz, 1H, O*H*), 1.42 (d, *J* = 6.6 Hz, 3H, C$H_3$/$6_B$)

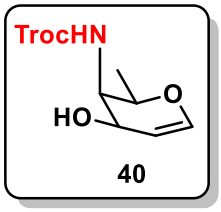
$^{13}$C NMR: (150 MHz, $CDCl_3$)

δ 145.1 ($1_B$), 102.6 ($2_B$), 72.6 ($5_B$), 65.2 ($3_B$), 63.1 ($4_B$), 17.9 (*C*$H_3$/$6_B$)

ESI-MS: Exact mass calcd. for $C_6H_9N_3O_2^+$ $[M+Na]^+$: 178.1, found 178.5

TLC: $R_f$ = 0.3 (hexane/EtOAc, 6:1)

**4-(2,2,2-trichloroethoxycarbonyl)amino-4,6-dideoxy-D-galactal (40):**




To a solution of compound **39** (2.0 g, 12.90 mmol, 1.0 equiv) in anhyd. THF (20 mL), was added LAH powder (0.98 g, 25.80 mmol, 2.0 equiv) in small portions at 0 °C. The reaction was allowed to stir at 0 ˚C for 1 h. After TLC showed complete reaction of the starting material, sat. $NH_4Cl$ (8 mL) was added slowly using a dropping funnel at 0 °C. After 1 h, reaction mixture was diluted with EtOAc:$H_2O$ (75:25 mL) and continually stirred for 15 min thereafter. The reaction mixture was then filtered through a bed of Celite® and then the aqueous layer was extracted with 5% methanol in $CH_2Cl_2$ (3 x 60 mL). The organic layers were combined, dried over $Na_2SO_4$, filtered and the solvent was removed under

reduced pressure to obtain the *cis*-amino alcohol **39a** (1.2 g, 72% yield). The *cis*-amino alcohol, **39a** (1.2 g, 9.30 mmol, 1.0 equiv) was then dissolved in anhyd. THF (25 mL) and $NaHCO_3$ (1.56 g, 18.6 mmol, 2.0 equiv) was added at 0 °C. Following the addition of $NaHCO_3$, Troc-Cl (1.54 mL, 11.6 mmol, 1.2 equiv) was added drop-wise and the reaction mixture was stirred at 0 ˚C for 1 h. After 1 h, the reaction mixture was filtered over a bed of Celite® and the solvent was removed under reduced pressure. Purification of the crude compound was achieved using silica gel chromatography (gradient method-hexane/EtOAc, 3:1) which afforded the desired compound **40** (1.78 g, 63% yield) as a colorless oil.

Data for compd **40**:

$^1$H NMR: (600 MHz, $CDCl_3$)

δ 6.38 (d, *J* = 6.2 Hz, 1H, $1_B$), 5.24 (d, *J* = 9.7 Hz, 1H, N*H*Troc), 4.87 (d, *J* = 12.0 Hz, 1H, C*H*H of Troc), 4.72 (d, *J* = 6.2 Hz, 1H, $2_B$), 4.68 (d, *J* = 12.0 Hz, 1H, C*H*H of Troc), 4.61 (d, *J* = 5.4 Hz, 1H, $3_B$), 4.19 (q, *J* = 6.4 Hz, 1H, $5_B$), 4.05 (m, 1H, $4_B$), 1.30 (d, *J* = 6.5 Hz, 3H, *C*$H_3$/$6_B$)

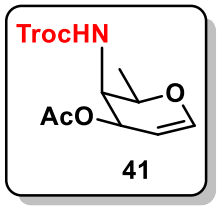
$^{13}$C NMR: (150 MHz, $CDCl_3$)

δ 156.2 (NH*C*(O)$OCH_2$), 145.1 ($1_B$), 103.5 ($2_B$), 95.5 (*C*$Cl_3$), 74.8 (*C*$H_2$), 72.8 ($5_B$), 64.4 ($3_B$), 52.1 ($4_B$), 17.1 (*C*$H_3$/$6_B$)

ESI-MS: Exact mass calcd. for $C_9H_{12}Cl_3NO_4^+$ $[M+Na]^+$: 325.9, found 325.9

TLC: $R_f$ = 0.3 (hexane/EtOAc, 3:1)

**3-*O*-acetyl-4-(2,2,2-trichloroethoxycarbonyl)amino-4,6-dideoxy-D-galactal (41):**



Compound **40** (1.78 g, 5.88 mmol, 1.0 equiv) was dissolved in anhyd. $CH_2Cl_2$ (40 mL) under an inert atmosphere and then anhyd. pyridine (1.42 mL, 17.64 mmol, 3.0 equiv) was added. The mixture was cooled to 0 °C and then acetyl chloride (0.51 mL, 7.05 mmol, 1.2 equiv) was added drop-wise, followed by stirring for 1 h at 0 ˚C. After TLC showed complete reaction of the starting material, the reaction mixture was diluted with $CH_2Cl_2$ (30 mL) and then an acid-base workup ensured to remove pyridine. The organic layer was separated, dried over $Na_2SO_4$, filtered and the organic solvent was removed under reduced pressure to obtain unpurified compound. Purification of the crude compound, using silica gel chromatography (gradient method-hexane/EtOAc, 4:1), afforded the desired compound **41** (1.85 g, 91% yield) as a colorless oil.

Data for compd **41**:

$^1$H NMR: (600 MHz, $CDCl_3$)

δ 6.46 (d, *J* = 6.3 Hz, 1H, $1_B$), 5.53 (d, *J* = 4.1 Hz, 1H, $3_B$), 5.16 (d, *J* = 9.8 Hz, 1H, N*H*Troc), 4.81 (d, *J* = 12.0 Hz, 1H, C*H*H-Troc), 4.73 (d, *J* = 12.0 Hz, 1H, CH*H*-Troc), 4.67 (dd, *J* = 6.4, 1.6 Hz, 1H, $2_B$), 4.24-4.20 (m, 2H, $4_B$, $5_B$), 2.01 (s, 3H, COC*H*$_3$), 1.31 (d, *J* = 6.5 Hz, 3H, C*H*$_3$/$6_B$)

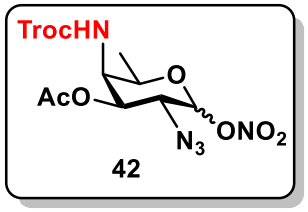
$^{13}$C NMR: (150 MHz, $CDCl_3$)

δ 170.5 (*C*OCH$_3$), 155.4 (NH*C*(O)OCH$_2$), 146.5 ($1_B$), 99.8 ($2_B$), 95.6 (*C*Cl$_3$), 74.6 (*C*H$_2$-Troc), 72.9 ($5_B$), 66.2 ($3_B$), 48.7 ($4_B$), 21.1 (CO*C*H$_3$), 16.9 (*C*H$_3$/$6_B$)

ESI-MS: Exact mass calcd. for $C_{11}H_{14}Cl_3NO_5^+$ $[M+2+Na]^+$: 369.9, found 369.8

TLC: $R_f$ = 0.4 (hexane/EtOAc, 4:1)

**3-*O*-acetyl-2-azido-4-(2,2,2-trichloroethoxycarbonyl)amino-2,4,6-trideoxy-D-galacto pyranosyl nitrate (42):**




42

To a solution of compound **41** (1.65 g, 4.78 mmol, 1.0 equiv) in anhyd. acetonitrile (25 mL) was added $NaN_3$ (0.47 g, 7.17 mmol, 1.5 equiv) and ceric ammonium nitrate (7.86 g, 14.35 mmol, 3.0 equiv) at -25 °C over a period of 30 min. The reaction mixture was stirred for 5 h at -25 °C and after TLC showed complete reaction of the starting material, the reaction mixture was diluted with EtOAc (100 mL) and washed with $H_2O$ (1 x 50 mL). The organic layer was separated, dried over $Na_2SO_4$, filtered and the solvent removed under reduced pressure to obtain unpurified compound.

Purification of the crude compound, using silica gel chromatography (gradient method-hexane/EtOAc, 4:1), afforded the desired compound **42** (1.57 g, 73% yield) with a *dr* ratio of 3.5:1 (*galacto*:*talo* = 3.5:1) as a white solid. ***Note:*** Galacto and talo diastereomers were inseparable using silica gel chromatography at this stage.

Data for compd **42**:

$^1$H NMR: (600 MHz, $CDCl_3$)

δ 6.26 (d, *J* = 4.4 Hz, 1H, $1_B$), 5.21 (m, 2H, $3_B$, N*H*Troc), 4.86 (d, *J* = 12.1 Hz, 1H, C*H*H-Troc), 4.71 (d, *J* = 12.1 Hz, 1H, CH*H*-Troc), 4.42 (q, *J* = 6.4 Hz, 1H, $5_B$), 4.34 (dd, *J* = 9.3, 3.5 Hz, 1H, $4_B$), 3.94 (dd, *J* = 11.4, 4.4 Hz, 1H, $2_B$), 2.07 (s, 3H, COC$H_3$), 1.26 (d, *J* = 6.4 Hz, 3H, C$H_3$/$6_B$)

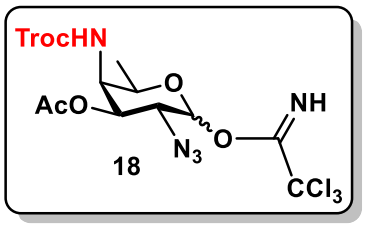
$^{13}$C NMR: (150 MHz, $CDCl_3$)

δ 169.9 (*C*$OCH_3$), 155.3 (NH*C*(O)$OCH_2$), 96.8 ($1_B$), 95.5 (*C*$Cl_3$), 74.7 (*C*$H_2$-Troc), 70.3 ($3_B$), 67.6 ($5_B$), 56.1 ($2_B$), 52.7 ($4_B$), 20.8 (CO*C*$H_3$), 16.4 (*C*$H_3$/$6_B$)

ESI-MS: Exact mass calcd. for $C_{11}H_{14}Cl_3N_5O_8^+$ $[M+Na]^+$: 471.9, found 471.6

TLC: $R_f$ = 0.5 (hexane/EtOAc, 4:1)

**3-*O*-acetyl-2-azido-4-(2,2,2-trichloroethoxycarbonyl)amino-2,4,6-trideoxy-D-galacto pyranosyl trichloroacetimidate (18):**



18

Compound **42** (0.5 g, 1.11 mmol, 1.0 equiv) was dissolved in anhyd. acetonitrile (15 mL), cooled to 0 °C and then DIPEA (0.24 mL, 1.34 mmol, 1.2 equiv) was added under an atmosphere of argon. A drop-wise addition of thiophenol (0.34 mL, 3.33 mmol, 3.0 equiv) at 0 ºC ensued and the reaction mixture was stirred for 40 min. The solvent was removed under reduced pressure and the residue was purified, using silica gel chromatography (gradient method-hexane/EtOAc, 3:1), to afford lactol **42a** (0.414 g) as a white solid. To a solution of lactol **42a** (0.41 g, 1.02 mmol, 1.0 equiv) and trichloroacetonitrile (0.21 mL, 2.05 mmol, 2.0 equiv) in anhyd. $CH_2Cl_2$ (6 mL) was added cat. DBU (23 µL, 0.15 mmol, 0.15 equiv) under an atmosphere of argon at 0 °C. The reaction mixture was allowed to stir for 1 h. The organic solvent was removed under reduced pressure and the unpurified compound was subjected to silica gel chromatography (gradient method-hexane/EtOAc,

6:1) to afford the desired galacto isomer **18** (410 mg, 67% yield) as a foamy white solid plus the undesired talo isomer **18a** (112 mg) as a white solid. A clear separation of the galacto and talo diastereomers was obtained in this step.

Data for compd **18**:

$^{1}$H NMR: (600 MHz, $CDCl_3$)

δ 8.76 (s, 1H, OC(N)*H*$CCl_3$), 6.42 (d, *J* = 3.7 Hz, 1H, $1_B$), 5.31-5.25 (m, 2H, $3_B$, N*H*Troc), 4.86 (d, *J* = 12.1 Hz, 1H, C*H*H of Troc), 4.71 (d, *J* = 12.1 Hz, 1H, CH*H* of Troc), 4.47 (q, *J* = 6.4 Hz, 1H, $5_B$), 4.37 (dd, *J* = 9.4, 1.9 Hz, 1H, $4_B$), 3.85 (dd, *J* = 11.1, 3.8 Hz, 1H, $2_B$), 2.08 (s, 3H, COC*H*$_3$), 1.24 (d, *J* = 6.5 Hz, 3H, C*H*$_3$/$6_B$)

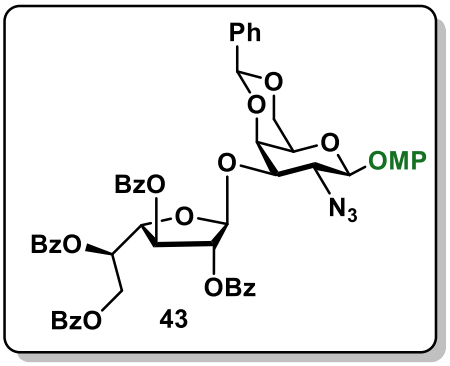
$^{13}$C NMR: (150 MHz, $CDCl_3$)

δ 170.2 (*C*O$CH_3$), 160.9 (O*C*(NH)$CCl_3$), 155.2 (NH*C*(O)O$CH_2$), 95.6 (*C*$Cl_3$), 94.4 ($1_B$), 74.7 (*C*$H_2$ of Troc), 70.3 ($3_B$), 67.5 ($5_B$), 57.1 ($2_B$), 52.9 ($4_B$), 20.9 (CO*C*$H_3$), 16.5 (*C*$H_3$/$6_B$)

ESI-MS: Exact mass calcd. for $C_{13}H_{15}Cl_6N_5O_6^+$ $[M+Na]^+$: 569.9, found 569.7

TLC: $R_f$ = 0.4 and 0.5 (hexane/EtOAc, 6:1)

**4-Methoxyphenyl 2,3,5,6-tetra-*O*-benzoyl-β-D-galactofuranosyl-(1→3)-2-azido-4,6-*O*-benzylidene-2-deoxy-β-D-galactopyranoside (43):**




To a solution of acceptor **20** (0.5 g, 1.25 mmol, 1.0 equiv) and donor **19** (1.11 g, 1.5 mmol, 1.2 equiv) in anhyd. $CH_2Cl_2$ (50 mL) was added fresh, flamed-dried, activated 4 Å molecular sieves (1 g) and the reaction was flushed three times with argon. The reaction mixture was stirred for about 45 min at room temperature, then cooled to -40 °C. TMSOTf (12 μL, 0.06 mmol, 0.05 equiv) in 0.1 mL anhyd. $CH_2Cl_2$ was then added drop-wise. The reaction mixture was stirred at -40 °C for about 30 min and after TLC showed complete reaction of starting material, the reaction mixture was quenched with triethylamine (0.5 mL) and filtered over a bed of Celite®. The organic solvent was removed under reduced pressure and purification of the compound was conducted using silica gel chromatography (gradient method-hexane/EtOAc, 3:2) affording the desired compound **43** (1.09 g, 89% yield) as a colorless oil.

Data for compd **43**:

$^1$H NMR: (600 MHz, $CDCl_3$)

δ 8.03 (dd, *J* = 8.2, 1.2 Hz, 2H, Ar-*H*), 7.96-7.90 (m, 6H, Ar-*H*), 7.53-7.49 (m, 4H, Ar-*H*), 7.44 (d, *J* = 7.7 Hz, 2H, Ar-*H*), 7.35 (t, *J* = 7.7 Hz, 2H, Ar-*H*), 7.30-7.26 (m, 6H, Ar-*H*), 7.22 (t, *J* = 7.4 Hz, 1H, Ar-*H*), 7.12-7.07 (m, 4H, Ar-*H*), 6.83-6.82 (m, 2H, Ar-*H*) 6.07-

6.06 (m, 1H, $5_D$), 5.66 (s, 1H, $3_D$), 5.65 (s, 1H, $1_D$), 5.59 (s, 1H, $2_D$) 5.50 (s, 1H, PhC*H*/benzylidene), 4.75-4.68 (m, 4H, $1_A$, $4_D$, $6_D$, $6_D$), 4.31-4.28 (m, 2H, $4_A$, $6_A$), 4.16 (dd, *J* = 10.4, 8.2 Hz, 1H, $2_A$), 3.93 (d, *J* = 6.2 Hz, 1H, $6_A$), 3.77 (s, 3H, OC$H_3$), 3.69 (dd, *J* = 10.4, 3.5 Hz, 1H, $3_A$), 3.45 (s, 1H, $5_A$)

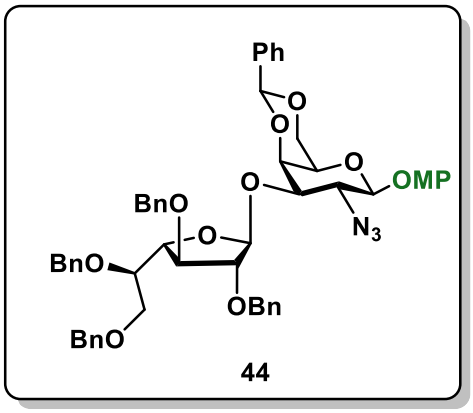
$^{13}$C NMR: (150 MHz, $CDCl_3$)

δ 166.4 (*C*OPh), 165.8 (*C*OPh), 165.7 (*C*OPh), 165.4 (*C*OPh), 155.7 (Ar-*C*), 151.1 (Ar-*C*), 137.5 (Ar-*C*), 133.6 (Ar-*C*), 133.5 (Ar-*C*), 133.5 (Ar-*C*), 133.4 (Ar-*C*), 130.1 (Ar-*C*), 130.0 (Ar-*C*), 129.8 (Ar-*C*), 129.5 (Ar-*C*), 129.4 (Ar-*C*), 129.0 (Ar-*C*), 128.9 (Ar-*C*), 128.6 (Ar-*C*), 128.6 (Ar-*C*), 128.5 (Ar-*C*), 128.5 (Ar-*C*), 128.2 (Ar-*C*), 126.1 (Ar-*C*), 119.2 (Ar-*C*), 114.6 (Ar-*C*), 107.4 ($1_D$), 101.9 ($1_A$), 100.8 (Ph*C*H/benzylidene), 82.5 ($4_D$), 82.0 ($2_D$), 77.8 ($3_D$), 77.5 ($3_A$), 74.8 ($4_A$), 70.3 ($5_D$), 68.9 ($6_A$), 66.6 ($5_A$), 63.4 ($6_D$), 61.7 ($2_A$), 55.7 (O*C*$H_3$)

ESI-MS: Exact mass calcd. for $C_{54}H_{47}N_3O_{15}^+$ $[M+Na]^+$: 1000.3, found 1000.4

TLC: $R_f$ = 0.5 (hexane/EtOAc, 3:2)

**4-Methoxyphenyl 2,3,5,6-tetra-*O*-benzyl-β-D-galactofuranosyl-(1→3)-2-azido-4,6-*O*-benzylidene-2-deoxy-β-D-galactopyranoside (44):**




44

Compound **43** (0.5 g, 0.51 mmol, 1.0 equiv) was dissolved in methanol (25 mL) and then freshly prepared 1 M NaOMe (3 mL, pH = ~9) was added. The reaction mixture was then stirred for 2 h at room temperature. Amberlite® resin ($H^+$) was added to the reaction mixture and stirred at room temperature until pH = 6. The mixture was then filtered over a cotton plug, and the solvent was removed under reduced pressure; dried under high vacuum for 1 h to obtain the unpurified compound **43a** (0.26 g). Unpurified compound **43a** (0.26 g, 0.46 mmol, 1.0 equiv) was dissolved in anhyd. DMF (5 mL) and NaH (0.15 g, 3.71 mmol, 8.0 equiv) was added at 0 °C in small portions followed by stirring for 15 min. Benzyl bromide (0.44 mL, 3.71 mmol, 8.0 equiv) was then added drop-wise at 0 °C and the reaction was stirred at room temperature for 5 h. After TLC showed complete reaction of the starting material, $H_2O$ (20 mL) was added slowly at 0 ºC. DMF was then evaporated under reduced pressure and the residue was diluted with EtOAc (60 mL). The reaction mixture was then washed with cold $H_2O$ (2 x 25 mL), dried over $Na_2SO_4$, filtered and the organic solvent was removed under reduced pressure. Purification was conducted using silica gel chromatography (gradient method-hexane/EtOAc, 2.5:1) to afford the desired compound **44** (0.41 g, 87% yield) as a colorless oil.

Data for compd **44**:

$^{1}$H NMR: (600 MHz, $CDCl_3$)

δ 7.47 (d, *J* = 7.6 Hz, 2H, Ar-*H*), 7.36-7.34 (m, 4H, Ar-*H*), 7.32-7.26 (m, 13H, Ar-*H*), 7.25-7.23 (m, 4H, Ar-*H*), 7.17 (d, *J* = 6.8 Hz, 2H, Ar-*H*), 7.07-7.05 (m, 2H, Ar-*H*), 6.82-6.80 (m, 2H, Ar-*H*), 5.35 (s, 1H, PhC*H*), 5.31 (s, 1H, $1_D$), 4.72-4.66 (m, 3H, $1_A$, PhC*H*H, PhCH*H*), 4.52 (t, *J* = 11.9 Hz, 2H, PhC*H*H, PhCH*H*), 4.45-4.40 (m, 3H, PhCH*H*, PhCH*H*, PhCH*H*), 4.30 (d, *J* = 3.5 Hz, 1H, $4_A$), 4.25-4.17 (m, 4H, PhCH*H*, $4_D$, $6_A$, $2_D$), 4.06 (dd, *J* = 10.4, 8.1 Hz, 1H, $2_A$), 3.96 (dd, *J* = 7.9, 3.4 Hz, 1H, $3_D$), 3.78 (s, 3H, OC$H_3$), 3.75-3.73 (m, 1H, $5_D$), 3.63-3.56 (m, 3H, $6_D$, $6_D$, $6_A$), 3.53 (dd, *J* = 10.4, 3.5 Hz, 1H, $3_A$), 3.31 (s, 1H, $5_A$)

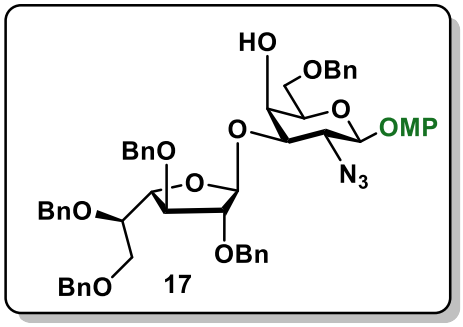
$^{13}$C NMR: (150 MHz, $CDCl_3$)

δ 155.2 (Ar-*C*), 151.2 (Ar-*C*), 138.5 (Ar-*C*), 138.2 (Ar-*C*), 137.9 (Ar-*C*), 137.8 (Ar-*C*), 137.5 (Ar-*C*), 128.9 (Ar-*C*), 128.6 (Ar-*C*), 128.5 (Ar-*C*), 128.4 (Ar-*C*), 128.4 (Ar-*C*), 128.2 (Ar-*C*), 128.1 (Ar-*C*), 128.0 (Ar-*C*), 127.8 (Ar-*C*), 127.8 (Ar-*C*), 127.8 (Ar-*C*), 127.6 (Ar-*C*), 126.3 (Ar-*C*), 119.2 (Ar-*C*), 114.5 (Ar-*C*), 108.3 ($1_D$), 101.9 ($1_A$), 100.7 (PhCH/benzylidene), 88.3 ($2_D$), 83.4 ($3_D$), 81.0 ($4_D$), 78.3 ($3_A$), 77.2 ($5_D$), 74.8 ($4_A$), 73.6 (Ph$CH_2$), 73.5 (Ph$CH_2$), 72.2 (Ph$CH_2$), 72.0 (Ph$CH_2$), 70.7 ($6_D$), 68.8 ($6_A$), 66.9 ($5_A$), 61.4 ($2_A$), 55.7 (O$CH_3$)

ESI-MS: Exact mass calcd. for $C_{54}H_{55}N_3O_{11}^{+}$ $[M+H+Na]^{+}$: 945.4, found 945.0

TLC: $R_f$ = 0.4 (hexane/EtOAc, 2.5:1)

**4-Methoxyphenyl 2,3,5,6-tetra-*O*-benzyl-β-D-galactofuranosyl-(1→3)-2-azido-6-*O*-benzyl-2-deoxy-β-D-galactopyranoside (17):**




To a solution of compound **44** (1.85 g, 2.01 mmol, 1.0 equiv) and triethyl silane (1.05 mL, 6.63 mmol, 3.3 equiv) in anhyd $CH_2Cl_2$ (40 mL) were added freshly activated 4 Å molecular sieves (2 g) and the reaction flask was flushed with argon three times. The reaction mixture was then stirred for 1 h at room temperature, cooled to -78 °C and TfOH (0.53 mL, 6.03 mmol, 3.0 equiv) was added drop-wise over a period of 40 min. The reaction mixture was then stirred at -78 °C for 5 h and after TLC showed complete reaction of starting material, methanol (3 mL) was added drop-wise, followed by triethyl amine (2 mL). The reaction mixture was then filtered over a bed of Celite® and the organic solvent was removed under reduced pressure. Purification of the crude compound, using silica gel chromatography (gradient method-hexane/EtOAc, 3:1), afforded the desired compound **17** (1.54 g, 83% yield) as a white solid.

Data for compd **17**:

$^1$H NMR: (600 MHz, $CDCl_3$)

δ 7.38-7.35 (m, 4H, Ar-*H*), 7.34-7.26 (m, 19H, Ar-*H*), 7.21 (d, *J* = 7.1 Hz, 2H, Ar-*H*), 7.08 (d, *J* = 8.9 Hz, 2H, Ar-*H*), 6.81 (d, *J* = 8.9 Hz, 2H, Ar-*H*),

5.31 (s, 1H, $1_D$), 4.70 (d, *J* = 8.2 Hz, 1H, $1_A$), 4.66 (d, *J* = 11.9 Hz, 1H, PhC*H*H), 4.63 (d, *J* = 11.8 Hz, 1H, PhCH*H*), 4.56-4.45 (m, 6H, PhC*HH*), 4.40 (d, *J* = 11.9 Hz, 1H, PhCH*H*), 4.33 (d, *J* = 11.6 Hz, 1H, PhCH*H*), 4.27 (dd, *J* = 6.8, 3.7 Hz, 1H, $4_D$), 4.16 (d, *J* = 3.4 Hz, 1H, $2_D$), 4.06 (d, *J* = 3.1 Hz, 1H, $4_A$), 4.05 (dd, *J* = 6.8, 3.5 Hz, 1H, $3_D$), 3.93 (t, *J* = 9.0 Hz, 1H, $2_A$), 3.75-3.70 (m, 3H, $5_D$, $6_A$, $6_A$), 3.70-3.65 (m, 1H, $5_A$), 3.61-3.55 (m, 2H, $6_D$, $6_D$), 3.49 (dd, *J* = 10.1, 3.2 Hz, 1H, $3_A$)

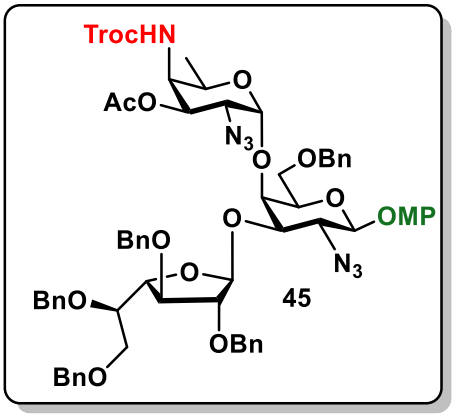
$^{13}$C NMR: (150 MHz, $CDCl_3$)

δ 155.6 (Ar-*C*), 151.3 (Ar-*C*), 138.2 (Ar-*C*), 138.1 (Ar-*C*), 137.9 (Ar-*C*), 137.6 (Ar-*C*), 137.4 (Ar-*C*), 128.6 (Ar-*C*), 128.5 (Ar-*C*), 128.5 (Ar-*C*), 128.4 (Ar-*C*), 128.3 (Ar-*C*), 128.1 (Ar-*C*), 128.1 (Ar-*C*), 128.0 (Ar-*C*), 127.9 (Ar-*C*), 127.8 (Ar-*C*), 127.8 (Ar-*C*), 127.8 (Ar-*C*), 127.7 (Ar-*C*), 127.7 (Ar-*C*), 118.7 (Ar-*C*), 114.6 (Ar-*C*), 107.6 ($1_D$), 101.6 ($1_A$), 88.0 ($2_D$), 82.1 ($3_D$), 81.7 ($4_D$), 79.5 ($3_A$), 75.7 ($5_D$), 74.1 ($5_A$), 73.7 (Ph*C*$H_2$), 73.5 (Ph*C*$H_2$), 73.3 (Ph*C*$H_2$), 72.3 (Ph*C*$H_2$), 72.2 (Ph*C*$H_2$), 69.7 ($6_D$), 69.5 ($6_A$), 67.8 ($4_A$), 62.2 ($2_A$), 55.7 (O*C*$H_3$)

ESI-MS: Exact mass calcd. for $C_{54}H_{57}N_3O_{11}^+$ $[M+Na]^+$: 946.4, found 946.6

TLC: $R_f$ = 0.5 (hexane/EtOAc, 3:1)

**4-methoxyphenyl 3-*O*-acetyl-2-azido-4-(2,2,2-trichloroethoxycarbonyl)amino-2,4,6-trideoxy-α-D-galacto pyranosyl-(1→4)-[ 2,3,5,6-tetra-*O*-benzyl-β-D-galactofuranosyl-(1→3)]-2-azido-6-*O*-benzyl-2-deoxy-β-D-galactopyranoside (45):**




To a solution of acceptor **17** (0.15 g, 0.16 mmol, 1.0 equiv) and donor **18** (0.18 g, 0.33 mmol, 2.0 equiv) in anhyd. $CH_2Cl_2$:ether (15 mL:8 mL), freshly dried 4 Å molecular sieves (0.25 g) were added. The reaction flask was flushed with argon for three times and the reaction mixture was stirred for 1 h at room temperature. TMSOTf (12 µL, 0.05 mmol, 0.3 equiv, in 0.2 mL $CH_2Cl_2$) was added drop-wise and the reaction was stirred for 20 min at room temperature. After TLC showed complete reaction of starting material, aq. $NaHCO_3$ (5 mL) was added. The reaction mixture was then filtered over a bed of Celite®, diluted with $CH_2Cl_2$ (50 mL), washed with water (1 x 20 mL) and the organic layer was separated out. The organic solvent was dried over $Na_2SO_4$, filtered and the organic solvent was removed under reduced pressure. Purification ensued using silica gel chromatography (gradient method-hexane/EtOAc, 4:1) to afford the desired compound **45** (0.134 g, 63% yield) as a colorless liquid.

Data for compd **45**:

$^1$H NMR: (600 MHz, $CDCl_3$)

δ 7.41-7.26 (m, 21H, Ar-*H*), 7.25-7.24 (m, 2H, Ar-*H*), 7.18 (d, *J* = 7.4 Hz, 2H, Ar-*H*), 7.05-7.02 (m, 2H, Ar-*H*), 6.82-6.79 (m, 2H, Ar-*H*), 5.38 (d, *J* =

1.4 Hz, 1H, $1_D$), 5.15 (dd, *J* = 11.2, 3.8 Hz, 1H, $3_B$), 4.93 (d, *J* = 9.7 Hz, 1H, N*H*Troc), 4.91 (d, *J* = 12.1 Hz, 1H, CH*H* of Troc), 4.79 (d, *J* = 11.9 Hz, 1H, PhCH*H*), 4.75 (d, *J* = 3.7 Hz, 1H, $1_B$), 4.73 (d, *J* = 7.9 Hz, 1H, $1_A$), 4.71 (d, *J* = 12.1 Hz, 1H, C*H*H of Troc), 4.64 (d, *J* = 11.8 Hz, 1H, PhC*H*H), 4.56-4.43 (m, 5H, PhC*H*H, PhCH*H*, PhC*H*H, PhCH*H*, PhC*H*H), 4.39-4.33 (m, 3H, $5_B$, PhCH*H*, PhC*H*H), 4.29-4.27 (m, 2H, $3_D$, PhCH*H*), 4.13 (ddd, *J* = 9.7, 3.7, 1.6 Hz, 1H, $4_B$), 4.08-4.05 (m, 2H, $2_D$, $5_A$), 3.96 (d, *J* = 2.6 Hz, 1H, $4_A$), 3.90-3.87 (m, 2H, $2_A$, $6_A$), 3.77 (s, 3H, OC$H_3$), 3.75-3.73 (m, 1H, $4_D$), 3.67-3.58 (m, 4H, $5_D$, $6_A$, $6_D$, $6_D$), 3.52 (dd, *J* = 11.0, 2.7 Hz, 1H, $3_A$), 2.79 (dd, *J* = 11.3, 3.7 Hz, 1H, $2_B$), 2.04 (s, 3H, COC$H_3$), 0.99 (d, *J* = 6.5 Hz, 3H, C$H_3$/$6_B$)

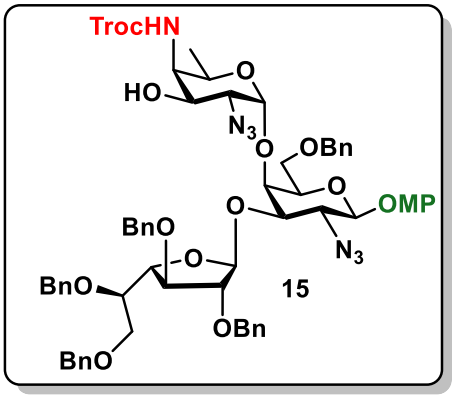
$^{13}$C NMR: (150 MHz, $CDCl_3$)

δ 170.2 (*C*O$CH_3$), 155.6 (Ar-*C*), 155.1 (Ar-*C*), 151.2 (Ar-*C*), 138.4 (Ar-*C*), 138.1 (Ar-*C*), 137.9 (Ar-*C*), 137.8 (Ar-*C*), 137.3 (Ar-*C*), 128.7 (Ar-*C*), 128.6 (Ar-*C*), 128.5 (Ar-*C*), 128.4 (Ar-*C*), 128.3 (Ar-*C*), 128.2 (Ar-*C*), 128.1 (Ar-*C*), 128.0 (Ar-*C*), 127.9 (Ar-*C*), 127.8 (Ar-*C*), 127.5 (Ar-*C*), 118.6 (Ar-*C*), 114.6 (Ar-*C*), 108.5 ($1_D$), 102.2 ($1_A$), 98.4 ($1_B$), 95.8 (*C*$Cl_3$), 87.3 ($2_D$), 82.3 ($5_A$), 80.1 ($3_D$), 77.4 ($3_A$), 77.0 ($4_A$), 75.1 ($4_D$), 74.6 (*C*$H_2$ of Troc), 74.1 ($5_D$), 73.5 (Ph*C*$H_2$), 73.4 (Ph*C*$H_2$), 73.2 (Ph*C*$H_2$), 72.2 (Ph*C*$H_2$), 72.0 (Ph*C*$H_2$), 70.6 ($3_B$), 69.7 ($6_D$), 67.7 ($6_A$), 64.7 ($5_B$), 62.9 ($2_A$), 58.4 ($2_B$), 55.7 (O*C*$H_3$), 53.2 ($4_B$), 21.0 (CO*C*$H_3$), 16.6 ($6_B$/*C*$H_3$)

ESI-MS: Exact mass calcd. for $C_{65}H_{70}Cl_3N_7O_{16}^+$ $[M+H+Na]^+$: 1333.4, found 1333.0

TLC: $R_f$ = 0.5 (hexane/EtOAc, 4:1)

**4-methoxyphenyl 2-azido-4-(2,2,2-trichloroethoxycarbonyl)amino-2,4,6-trideoxy-α-D-galacto pyranosyl-(1→4)-[ 2,3,5,6-tetra-*O*-benzyl-β-D-galactofuranosyl-(1→3)]-2-azido-6-*O*-benzyl-2-deoxy-β-D-galactopyranoside (15):**




To a solution of compound **45** (0.23 g, 0.18 mmol, 1.0 equiv) in methanol:$CH_2Cl_2$ (10 mL:5 mL) was added *p*-TsOH (0.32 g, 1.8 mmol, 10 equiv) and the reaction mixture was heated to 60 °C for 24 h. After TLC showed complete reaction of starting material, the solvents were removed under reduced pressure. The residue was then diluted with $CH_2Cl_2$ (50 mL) and washed with aq $NaHCO_3$ (1 x 20 mL). The organic layer was separated, dried over $Na_2SO_4$, filtered, and solvent was removed under reduced pressure. Purification ensued using silica gel chromatography (gradient method-hexane/EtOAc, 4:1) to afford the desired compound **15** (0.16 g, 72% yield) as a colorless liquid.

Data for compd **15**:

$^1$H NMR: (600 MHz, $CDCl_3$)

δ 7.38-7.27 (m, 21H, Ar-*H*), 7.25-7.24 (m, 2H, Ar-*H*), 7.19 (d, *J* = 7.5 Hz, 2H, Ar-*H*), 7.05-7.03 (m, 2H, Ar-*H*), 6.81-6.79 (m, 2H, Ar-*H*), 5.37 (d, *J* =

1.4 Hz, 1H, $1_D$), 4.93 (d, *J* = 9.5 Hz, 1H, N*H*Troc), 4.87 (d, *J* = 12 Hz, 1H, C*H*H-Troc), 4.76-4.74 (m, 3H, $1_B$, CH*H* of Troc, PhC*H*H), 4.72 (d, *J* = 7.9 Hz, 1H, $1_A$), 4.65 (d, *J* = 11.8 Hz, 1H, PhCH*H*), 4.55-4.48 (m, 4H, PhC*H*H, PhCH*H*, PhC*H*H, PhCH*H*), 4.44 (d, *J* = 11.3 Hz, 1H, PhC*H*H), 4.38-4.34 (m, 2H, PhCH*H*, PhC*H*H), 4.30-4.26 (m, 3H, $3_D$, $5_B$, PhCH*H*), 4.13-4.11 (m, 1H, $3_B$), 4.09-4.05 (m, 2H, $2_D$, $5_A$), 3.96 (d, *J* = 2.5 Hz, 1H, $4_A$), 3.90-3.87 (m, 2H, $4_B$, $6_A$), 3.83 (dd, *J* = 10.8, 7.9 Hz, 1H, $2_A$), 3.77 (s, 3H, OC*H*$_3$), 3.76-3.73 (m, 1H, $4_D$), 3.68-3.58 (m, 4H, $5_D$, $6_D$, $6_D$, $6_A$), 3.50 (dd, *J* = 10.9, 2.6 Hz, 1H, $3_A$), 2.51 (dd, *J* = 10.7, 3.5 Hz, 1H, $2_B$), 2.36 (d, *J* = 4.6 Hz, 1H, O*H*), 1.04 (d, *J* = 6.5 Hz , 3H, C*H*$_3$/$6_B$)

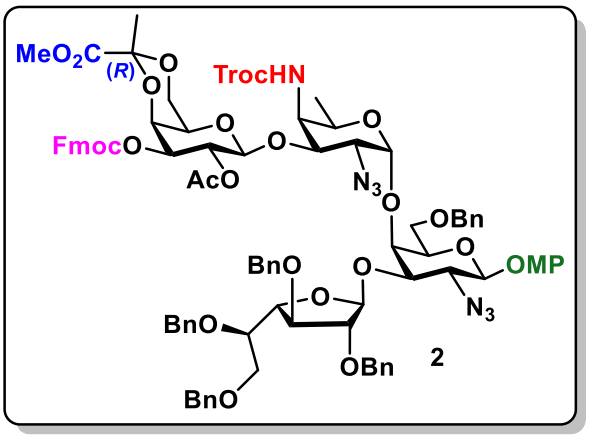
$^{13}$C NMR: (150 MHz, $CDCl_3$)

δ 156.3 (*C*O of Troc), 155.7 (Ar-*C*), 151.1 (Ar-*C*), 138.4 (Ar-*C*), 138.2 (Ar-*C*), 138.0 (Ar-*C*), 137.9 (Ar-*C*), 137.5 (Ar-*C*), 128.6 (Ar-*C*), 128.5 (Ar-*C*), 128.4 (Ar-*C*), 128.2 (Ar-*C*), 128.1 (Ar-*C*), 128.1 (Ar-*C*), 128.0 (Ar-*C*), 127.9 (Ar-*C*), 127.9 (Ar-*C*), 127.9 (Ar-*C*), 127.8 (Ar-*C*), 127.6 (Ar-*C*), 118.7 (Ar-*C*), 114.6 (Ar-*C*), 108.5 ($1_D$), 102.3 ($1_A$), 98.6 ($1_B$), 95.5 (*C*$Cl_3$), 87.4 ($2_D$), 82.3 ($5_A$), 80.5 ($3_D$), 77.6 ($3_A$), 77.0 ($4_A$), 75.2 ($4_D$), 74.9 (*C*$H_2$ of Troc), 74.3 ($5_D$), 73.5 (Ph*C*$H_2$), 73.4 (Ph*C*$H_2$), 73.2 (Ph*C*$H_2$), 72.3 (Ph*C*$H_2$), 71.9 (Ph*C*$H_2$), 69.7 ($6_D$), 69.0 ($3_B$), 68.1 ($6_A$), 64.9 ($5_B$), 63.1 ($2_A$), 61.6 ($2_B$), 56.2 ($4_B$), 55.7 (O*C*$H_3$), 16.8 ($6_B$/*C*$H_3$)

ESI-MS: Exact mass calcd. for $C_{63}H_{68}Cl_3N_7O_{15}^+$ $[M+2+Na]^+$: 1292.4, found 1292.3

TLC: $R_f$ = 0.3 (hexane/EtOAc, 4:1)

**4-methoxyphenyl 3-*O*-Fluorenylmethyloxycarbonyl-2-*O*-benzoyl-4,6-*O*-[1-(*R*)-(methoxycarbonyl)-ethylidene]-β-D-galactopyranoside-(1→3)- 2-azido-4-(2,2,2-trichloroethoxycarbonyl)amino-2,4,6-trideoxy-α-D-galacto pyranosyl-(1→4)-[ 2,3,5,6-tetra-*O*-benzyl-β-D-galactofuranosyl-(1→3)]-2-azido-6-*O*-benzyl-2-deoxy-β-D-galactopyranoside (2):**




To a solution of acceptor **15** (35 mg, 0.028 mmol, 1.0 equiv) and donor **16** (31 mg, 0.050 mmol, 1.8 equiv) in anhyd. $CH_2Cl_2$ (3 mL), freshly dried 4 Å molecular sieves (80 mg) were added and the reaction flask was flushed with argon three times. The reaction mixture was then stirred for 1 h at room temperature. NIS (13 mg, 0.056 mmol, 2.0 equiv) was added and the reaction was allowed to cool to 0 °C at which point TMSOTf (2 μL in 0.1 mL $CH_2Cl_2$, 0.006 mmol, 0.2 equiv) was added dropwise. The reaction was then stirred for 30 min. After TLC showed complete reaction of starting material, aq $NaHCO_3$ (2 mL) was added to the reaction mixture, it was filtered over a bed of Celite®, washed with $CH_2Cl_2$ (1 x 25 mL) and water (1 x 10 mL). The organic layer was separated and washed with aq $Na_2S_2O_3$ solution (1 x 10 mL). The organic solvent was then dried over $Na_2SO_4$, filtered and removed under reduced pressure. The unpurified compound was subjected to

silica gel chromatography (gradient method-hexane/EtOAc, 3:1) and the desired compound **2** (35 mg, 72% yield) was isolated as a colorless oil.

Data for compd **2**:

$^{1}$H NMR: (600 MHz, $CDCl_3$)

δ 7.77 (d, *J* = 7.6 Hz, 2H, Ar-*H*), 7.64-7.62 (m, 2H, Ar-*H*), 7.41 (t, *J* = 7.4 Hz, 2H, Ar-*H*), 7.37-7.24 (m, 26H, Ar-*H*), 7.18 (d, *J* = 6.6 Hz, 2H, Ar-*H*), 7.05-7.02 (m, 2H, Ar-*H*), 6.81-6.79 (m, 2H, Ar-*H*), 5.45 (dd, *J* = 10.3, 7.9 Hz, 1H, $2_C$), 5.35 (d, *J* = 1.4 Hz, 1H, $1_D$), 5.09 (d, *J* = 12.1 Hz, 1H, C*H*H-Troc), 4.87 (d, *J* = 9.5 Hz, 1H, N*H*Troc), 4.74-4.67 (m, 4H, $1_B$, $1_A$, $3_C$, PhC*H*H), 4.63-4.61 (m, 2H, $1_C$, PhCH*H*), 4.55-4.46 (m, 5H, CH*H*-Troc, PhC*H*H, PhCH*H*, PhC*H*H, PhCH*H*), 4.44-4.42 (m, 3H, $4_C$, PhC*H*H, PhCH*H*), 4.37-4.26 (m, 6H, $4_D$, PhC*H*H, PhCH*H*, PhC*H*H, PhCH*H*, C*H* of Fmoc), 4.19-4.10 (m, 4H, $5_B$, $4_B$, $6_C$, $2_D$), 4.06 (dd, *J* = 7.9, 4.6 Hz, 1H, $3_D$), 4.01 (dd, *J* = 10.9, 4.8 Hz, 1H, $3_B$), 3.96 (d, *J* = 2.5 Hz, 1H, $4_A$), 3.91-3.85 (m, 2H, $6_C$, $6_A$), 3.81 (dd, *J* = 10.8, 7.9 Hz, 1H, $2_A$), 3.76 (s, 3H, OC$H_3$ of OMP group), 3.75-3.72 (m, 1H, $5_D$), 3.68-3.66 (m, 2H, $5_A$, $6_A$), 3.64-3.56 (m, 5H, $6_D$, $6_D$, COOC$H_3$), 3.49 (dd, *J* = 10.9, 2.4 Hz, 1H, $3_A$), 3.41 (s, 1H, $5_C$), 2.51 (dd, *J* = 10.9, 3.7 Hz, 1H, $2_B$), 2.08 (s, 3H, C$H_3$CO), 1.61 (s, 3H, pyruvate C$H_3$), 1.09 (d, *J* = 6.4 Hz, 3H, C$H_3$/$6_B$)

$^{13}$C NMR: (150 MHz, $CDCl_3$)

δ 170.3 (*C*$O_2CH_3$), 169.5 (*C*O$CH_3$), 155.7 (Ar-*C*), 155.2 (*C*$O_2$-Troc), 154.3 (*C*$O_2$-Fmoc), 151.1 (Ar-*C*), 143.5 (Ar-*C*), 143.2 (Ar-*C*), 141.4 (Ar-*C*),

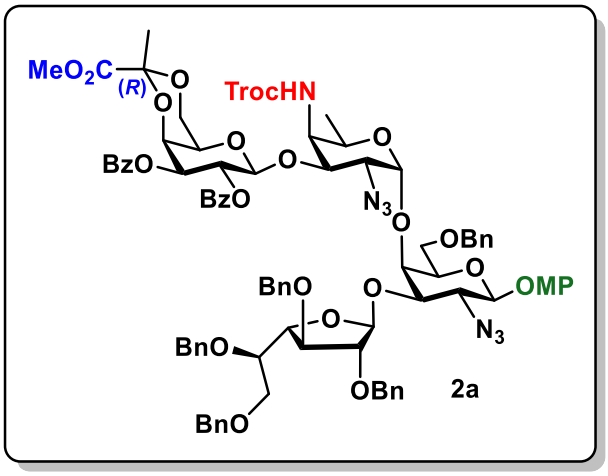
141.3 (Ar-*C*), 138.3 (Ar-*C*), 138.1 (Ar-*C*), 137.9 (Ar-*C*), 137.9 (Ar-*C*), 137.5 (Ar-*C*), 128.6 (Ar-*C*), 128.6 (Ar-*C*), 128.5 (Ar-*C*), 128.5 (Ar-*C*), 128.1 (Ar-*C*), 128.0 (Ar-*C*), 128.0 (Ar-*C*), 127.9 (Ar-*C*), 127.9 (Ar-*C*), 127.7 (Ar-*C*), 127.6 (Ar-*C*), 127.4 (Ar-*C*), 127.3 (Ar-*C*), 125.6 (Ar-*C*), 125.4 (Ar-*C*), 120.1 (Ar-*C*), 120.1 (Ar-*C*), 118.6 (Ar-*C*), 114.7 (Ar-*C*), 108.7 ($1_D$), 102.3 ($1_A$), 101.2 ($1_C$), 98.9 ($1_B$), 98.7 (pyruvate quaternary *C*), 95.9 (*C*$Cl_3$), 87.6 ($2_D$), 82.2 ($3_D$), 80.3 ($4_D$), 77.8 ($3_A$), 77.6 ($4_A$), 76.1 ($3_B$), 75.6 ($3_C$), 75.0 ($5_D$), 74.8 (*C*$H_2$ of Troc), 74.3 ($5_A$), 73.5 (Ph*C*$H_2$), 73.4 (Ph*C*$H_2$), 73.2 (Ph*C*$H_2$), 72.2 (Ph*C*$H_2$), 72.1 (Ph*C*$H_2$), 72.0 (Ph*C*$H_2$), 70.5 (Ph*C*$H_2$), 69.6 ($6_D$), 68.4 ($2_C$), 68.3 ($4_C$), 67.9 ($6_A$), 65.6 ($5_C$), 65.5 ($5_B$), 64.5 ($6_C$), 63.1 ($2_A$), 60.4 ($2_B$), 55.7 (O*C*$H_3$ of OMP), 54.8 ($4_B$), 52.5 ($CO_2$*C*$H_3$), 46.5 (*C*H of Fmoc), 25.7 (pyruvate *C*$H_3$), 21.0 (CO*C*$H_3$), 16.6 ($6_B$/*C*$H_3$)

HRMS: Exact mass calcd. for $C_{90}H_{94}Cl_3N_7O_{25}^+$ $[M+2+Na]^+$: 1802.5263, found 1802.5310

OR: $[\alpha]_D^{22.5}$ = +24.6 (C = 1.00 in $CHCl_3$)

TLC: $R_f$ = 0.4 (hexane/EtOAc, 3:1)

**4-methoxyphenyl 2,3-*O*-benzoyl-4,6-*O*-[1-(*R*)-(methoxycarbonyl)-ethylidene]-β-D-galactopyranoside-(1→3)- 2-azido-4-(2,2,2-trichloroethoxycarbonyl)amino-2,4,6-trideoxy-α-D-galacto pyranosyl-(1→4)-[ 2,3,5,6-tetra-*O*-benzyl-β-D-galactofuranosyl-(1→3)]-2-azido-6-*O*-benzyl-2-deoxy-β-D-galactopyranoside (2a):**




To a solution of acceptor **15** (0.08 g, 0.063 mmol, 1.0 equiv) and donor **26** (0.064 g, 0.114 mmol, 1.8 equiv) in anhyd. $CH_2Cl_2$ (6 mL) was added freshly dried 4 Å activated molecular sieves (60 mg). The reaction flask was flushed with argon three times and the mixture was allowed to stir for 30 min at room temperature. NIS (0.027 g, 0.14 mmol, 2.0 equiv) was added and the reaction was cooled to 0 °C. After a short period of time (5 min), TMSOTf (4 μL, 0.012 mmol, 0.2 equiv) in 0.1 mL $CH_2Cl_2$ was added drop-wise and the reaction was stirred for 30 min.

After TLC showed complete reaction of starting material, aq $NaHCO_3$ (5 mL) was added to the reaction mixture and then it was filtered over a bed of Celite®. The mixture was then washed with $CH_2Cl_2$ (1 x 20 mL) and water (1 x 10 mL). The organic layer was separated and washed with aq $Na_2S_2O_3$ solution (1 x 10 mL). The organic solvent was dried over $Na_2SO_4$, filtered and removed under reduced pressure. The residue was then subjected to silica gel chromatography (gradient method-hexane/EtOAc, 3:1) to afford the desired compound **2a** (0.075 g, 69% yield) as a white solid.

Data for compd **2a**:

$^{1}$H NMR: (600 MHz, $CDCl_3$)

δ 8.01-7.99 (m, 4H, Ar-*H*), 7.52 (t, *J* = 7.4 Hz, 1H, Ar-*H*), 7.42-7.27 (m, 24H, Ar-*H*), 7.22-7.17 (m, 6H, Ar-*H*), 7.06-7.03 (d, *J* = 9.1 Hz, 2H, Ar-*H*), 6.83-6.81 (d, *J* = 9.1 Hz, 2H, Ar-*H*), 5.80 (dd, *J* = 10.3, 8.0 Hz, 1H, $2_C$), 5.30 (d, *J* = 1.0 Hz, 1H, $1_D$), 5.14-5.09 (m, 2H, $3_C$, C*H*H of Troc), 4.84 (d, *J* = 8.0 Hz, 1H, $1_C$), 4.78 (d, *J* = 9.4 Hz, 1H, N*H*Troc), 4.73-4.69 (m, 3H, $1_A$, $1_B$, PhC*H*H), 4.61-4.57 (m, 2H, CH*H* of Troc, PhCH*H*), 4.54-4.46 (m, 3H, $4_C$, PhC*H*H, PhCH*H*), 4.42-4.40 (d, *J* = 11.3 Hz, 1H, PhC*H*H), 4.34-4.23 (m, 7H, $6_C$, $4_D$, PhCH*H*, PhC*H*H, PhCH*H*, PhC*H*H, PhCH*H*), 4.16-4.11 (m, 3H, $4_B$, $5_B$, $2_D$), 4.04-3.95 (m, 3H, $6_C$, $3_B$, $3_D$), 3.88 (d, *J* = 2.2 Hz, 1H, $4_A$), 3.78-3.76 (m, 4H, $2_A$, OC$H_3$ of OMP), 3.74-3.68 (m, 2H, $5_D$, $6_A$), 3.65 (s, 3H, $CO_2CH_3$), 3.64-3.52 (m, 5H, $5_C$, $5_A$, $6_D$, $6_D$, $6_A$), 3.46 (dd, *J* = 10.8, 2.3 Hz, 1H, $3_A$), 2.30 (dd, *J* = 10.9, 3.6 Hz, 1H, $2_B$), 1.59 (s, 3H, pyruvate C$H_3$), 1.08 (d, *J* = 6.4 Hz, 3H, C$H_3$/$6_B$)

$^{13}$C NMR: (150 MHz, $CDCl_3$)

δ 170.5 (*C*$O_2CH_3$), 166.2 (*C*OPh), 165.4 (*C*OPh), 155.7 (Ar-*C*), 155.2 (Ar-*C*), 151.1 (Ar-*C*), 138.2 (Ar-*C*), 138.1 (Ar-*C*), 138.0 (Ar-*C*), 137.5 (Ar-*C*), 133.4 (Ar-*C*), 133.1 (Ar-*C*), 130.0 (Ar-*C*), 129.9 (Ar-*C*), 129.8 (Ar-*C*), 129.5 (Ar-*C*), 128.6 (Ar-*C*), 128.6 (Ar-*C*), 128.5 (Ar-*C*), 128.5 (Ar-*C*), 128.4 (Ar-*C*), 128.4 (Ar-*C*), 128.4 (Ar-*C*), 128.2 (Ar-*C*), 128.1 (Ar-*C*), 128.1 (Ar-*C*), 128.0 (Ar-*C*), 127.9 (Ar-*C*), 127.9 (Ar-*C*), 127.8 (Ar-*C*), 127.8 (Ar-*C*), 127.7 (Ar-*C*), 127.6 (Ar-*C*), 118.5 (Ar-*C*), 114.7 (Ar-*C*),

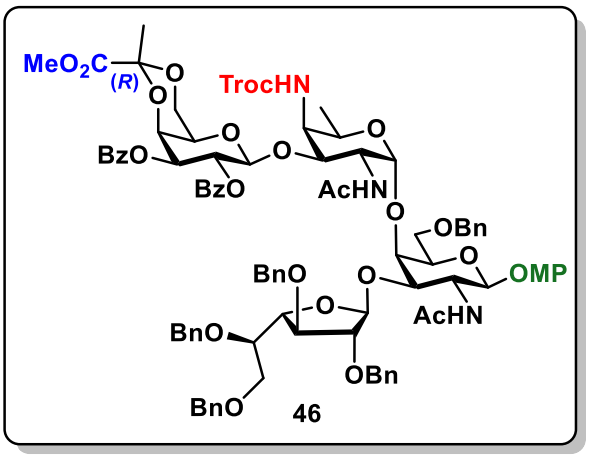
108.7 ($1_D$), 102.2 ($1_A$), 101.7 ($1_C$), 99.0 ($1_B$), 98.7 (pyruvate quaternary *C*), 95.9 (*C*$Cl_3$), 87.4 ($2_D$), 82.2 ($3_D$), 80.6 ($4_D$), 77.9 ($3_A$), 77.8 ($4_A$), 76.2 ($3_B$), 75.0 ($5_D$), 74.9 (*C*$H_2$ of Troc), 74.4 ($5_A$), 73.4 (Ph*C*$H_2$), 73.3 (Ph*C*$H_2$), 73.1 (Ph*C*$H_2$), 73.0 ($3_C$), 72.1 (Ph*C*$H_2$), 72.0 (Ph*C*$H_2$), 69.6 ($6_D$), 69.1 ($2_C$), 68.9 ($4_C$), 68.2 ($6_A$), 65.9 ($5_C$), 65.7 ($5_B$), 64.6 ($6_C$), 63.2 ($2_A$), 60.5 ($2_B$), 55.8 (O*C*$H_3$), 55.1 ($4_B$), 52.5 ($CO_2$*C*$H_3$), 25.7 (pyruvate *C*$H_3$), 16.7 ($6_B$/*C*$H_3$)

HRMS: Exact mass calcd. for $C_{87}H_{90}Cl_3N_7O_{24}^+$ $[M+H]^+$: 1722.5103, found 1722.5181

OR: $[\alpha]_D^{22.5}$ = +37.5 (C = 0.45 in $CHCl_3$)

TLC: $R_f$ = 0.4 (hexane/EtOAc, 3:1)

**4-methoxyphenyl 2,3-*O*-benzoyl-4,6-*O*-[1-(*R*)-(methoxycarbonyl)-ethylidene]-β-D-galactopyranoside-(1→3)- 2-acetamido-4-(2,2,2-trichloroethoxycarbonyl)amino-2,4,6-trideoxy-α-D-galacto pyranosyl-(1→4)-[ 2,3,5,6-tetra-*O*-benzyl-β-D-galactofuranosyl-(1→3)]-2-acetamido-6-*O*-benzyl-2-deoxy-β-D-galactopyranoside (46):**



To a solution of compound **2a** (0.07 g, 0.041 mmol, 1.0 equiv) in pyridine (0.7 mL) at 0 ºC was added thioacetic acid (0.6 mL) drop-wise over a period of 10 min and the reaction was allowed to stir at room temperature for approximately 24 h. The solvents were removed under vacuum and the unpurified compound was subjected to silica gel chromatography (gradient method-hexane/EtOAc, 1:1) giving the desired compound **46** (0.043 g, 61% yield) as a white solid.

Data for compd **46**:

$^1$H NMR: (600 MHz, $CDCl_3$)

δ 8.03-7.98 (m, 4H, Ar-*H*), 7.51 (t, *J* = 7.6 Hz, 1H, Ar-*H*), 7.38-7.22 (m, 25H, Ar-*H*), 7.20 (d, *J* = 6.8 Hz, 2H, Ar-*H*), 7.16 (d, *J* = 7.1 Hz, 2H, Ar-*H*), 6.91-6.87 (m, 2H, Ar-*H*), 6.78-6.76 (m, 2H, Ar-*H*), 5.91 (s, br, 1H, N*H*Ac), 5.81 (dd, *J* = 10.3, 7.9 Hz, 1H, $2_C$), 5.60 (d, *J* = 8.8 Hz, 1H, N*H*Troc), 5.37 (s, br, 1H, N*H*Ac), 5.26 (d, *J* = 2.1 Hz, 1H, $1_D$), 5.14 (dd, *J* = 10.3, 3.5 Hz, 1H, $3_C$), 5.05 (d, *J* = 12.1 Hz, 1H, C*H*H-Troc), 4.91 (d, *J* = 8.0 Hz, 1H, $1_C$), 4.81-4.79 (m, 2H, $1_B$, $1_A$), 4.64 (d, *J* = 11.6 Hz, 1H, PhC*H*H), 4.59 (d, *J* = 11.7 Hz, 1H, PhCH*H*), 4.50 (d, *J* = 3.5 Hz, 1H, $4_C$), 4.44-4.35 (m, 9H, $5_B$, $6_A$, CH*H* of Troc, PhC*H*H, PhCH*H*, PhC*H*H, PhCH*H*, PhC*H*H, PhCH*H*), 4.29-4.20 (m, 7H, $4_D$, $3_B$, $4_B$, $2_A$, $5_A$, PhC*H*H, PhCH*H*), 4.13-4.09 (m, 1H, $2_B$), 4.05-4.03 (m, 2H, $3_D$, $4_A$), 3.99-3.95 (m, 3H, $2_D$, $3_A$, $6_A$), 3.76 (s, 3H, $OCH_3$ of OMP), 3.68-3.66 (m, 1H, $5_D$), 3.64 (s, 3H, $CO_2CH_3$), 3.62-3.53 (m, 3H, $6_D$, $6_D$, $5_C$), 3.37-3.35 (m, 1H, $6_C$), 3.26-3.23 (t, *J* = 7.5 Hz, 1H, $6_C$),

1.90 (s, 3H, NHCOC$H_3$), 1.56 (s, 3H, pyruvate C$H_3$), 1.25 (s, 3H, NHCOC$H_3$), 1.08 (d, $J$ = 6.4 Hz, 3H, $6_B$/C$H_3$)

$^{13}$C NMR: (150 MHz, $CDCl_3$)

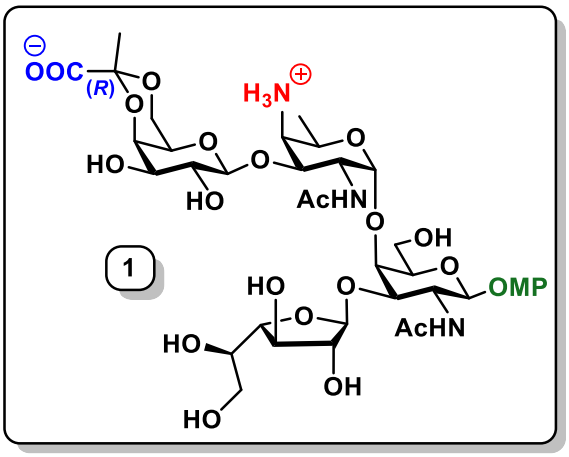
δ 170.8 (NH*C*O$CH_3$), 170.5 (NH*C*O$CH_3$), 170.2 (*C*$O_2CH_3$), 166.2 (*C*OPh), 165.1 (*C*OPh), 155.6 (Ar-*C*), 155.5 (Ar-*C*), 151.6 (Ar-*C*), 138.2 (Ar-*C*), 137.6 (Ar-*C*), 137.5 (Ar-*C*), 137.3 (Ar-*C*), 133.4 (Ar-*C*), 130.2 (Ar-*C*), 130.0(Ar-*C*), 129.9 (Ar-*C*), 129.6 (Ar-*C*), 129.4 (Ar-*C*), 128.8 (Ar-*C*), 128.7 (Ar-*C*), 128.6 (Ar-*C*), 128.5 (Ar-*C*), 128.5 (Ar-*C*), 128.4 (Ar-*C*), 128.4 (Ar-*C*), 128.3 (Ar-*C*), 128.1 (Ar-*C*), 128.0 (Ar-*C*), 127.9 (Ar-*C*), 127.8 (Ar-*C*), 127.7 (Ar-*C*), 118.4 (Ar-*C*), 114.7 (Ar-*C*), 107.6 ($1_D$), 101.4 ($1_A$, $1_C$), 98.7 (pyruvate quaternary carbon), 97.5 ($1_B$), 95.9 ($CCl_3$), 87.7 ($2_D$), 81.9 ($3_D$), 81.3 ($4_D$), 75.9 ($5_D$), 75.7 ($2_B$), 74.8 ($3_A$), 74.7 (*C*$H_2$ of Troc), 74.1 ($4_A$), 73.5 (Ph*C*$H_2$), 73.4 (Ph*C*$H_2$), 73.3 (Ph*C*$H_2$), 73.1 ($3_C$), 72.2 (Ph*C*$H_2$), 71.9 (Ph*C*$H_2$), 70.4 ($6_D$), 69.3 ($2_C$), 68.9 ($4_C$), 67.6 ($6_C$), 66.0 ($5_B$), 65.9 ($5_C$), 64.7 ($6_A$), 55.8 (OC$H_3$ of OMP), 52.9 ($3_B$, $4_B$), 52.5 ($CO_2CH_3$), 25.8 (pyruvate *C*$H_3$), 23.8 (NHCO*C*$H_3$), 22.5 (NHCO*C*$H_3$), 16.8 ($6_B$/*C*$H_3$)

HRMS: Exact mass calcd. for $C_{91}H_{98}Cl_3N_3O_{26}^+$ $[M+Na]^+$: 1776.5402, found 1776.5491

OR: $[\alpha]_D^{22.5}$ = +21.5 (C = 0.1 in $CHCl_3$)

TLC: $R_f$ = 0.4 (hexane/EtOAc, 1:1)

**4-methoxyphenyl 4,6-*O*-[1-(*R*)-(methoxycarbonyl)-ethylidene]-β-D-galactopyranoside-(1→3)-2-acetamido-4-amino-2,4,6-trideoxy-α-D-galacto pyranosyl-(1→4)-[β-D-galactofuranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-galactopyranoside (1):**




To a solution of compound **46** (18 mg, 0.02 mmol, 1.0 equiv) in THF:AcOH (1:0.6 mL) at 0 ºC was added activated Zn (30 mg). The ice bath was taken out and the reaction mixture was stirred to room temperature taking approximately 2 h. Furthermore, the reaction was cooled to 0 ºC and more activated Zn (15 mg) was added while continually stirring for another 2 h.

Aq $NaHCO_3$ (2 mL) was added to the reaction mixture, diluted with EtOAc (10 mL) and filtered over a bed of Celite®. The organic layer was washed with more aq $NaHCO_3$ (1 x 5 mL), dried over $Na_2SO_4$ and the solvent was removed under vacuum to obtain the unpurified amine **46a**. Amine **46a** was then dissolved in a mixture of EtOAc:MeOH:AcOH:$H_2O$ (2:8:1:2), and Pd/C (5 mg, 0.25 g/mmol) was added at room temperature. The reaction was then stirred under an atmosphere of hydrogen (balloon, 1 atm) for 36 h. The reaction was then filtered over a bed of Celite®, washed with MeOH

(2 x 5 mL) and finally the solvents were removed under reduced pressure to obtain unpurified compound **46b**. Crude **46b** was then dissolved in THF:$H_2O$ (1:4) and 1 M LiOH solution was added dropwise until pH = 11-12. The reaction was stirred at room temperature for 24 h and AcOH was added dropwise until pH = 7. THF was removed under vacuum and the reaction mixture was filtered through a cotton plug and washed with water (2 x 3 mL). The aqueous reaction mixture was frozen and then lyophilized to obtain unpurified compound in a powdered form. The compound was ultimately purified using a Bio-Gel® P-2 column (BIO-RAD, fine, 45-90 μm; was passed through twice) to obtain the desired zwitterionic tetrasaccharide **1** (5.2 mg, 56% yield over three steps) as a fluffy white solid.

Data for compd **1**:

$^1$H NMR: (600 MHz, $CDCl_3$)

δ 7.06 (d, *J* = 9.1 Hz, 2H, Ar-*H*), 6.96 (d, *J* = 9.1 Hz, 2H, Ar-*H*), 5.08 (d, *J* = 8.5 Hz, 1H, $1_A$), 5.05 (d, *J* = 3.2 Hz, 1H, $1_D$), 5.03 (d, *J* = 3.1 Hz, 1H, $1_B$), 4.66 (s, br, 1H, $5_B$), 4.59 (d, *J* = 7.8 Hz, 1H, $1_C$), 4.38 (t, *J* = 10.3 Hz, 2H, $2_A$, $3_B$), 4.23 (dd, *J* = 11.5, 3.7 Hz, 1H, $2_B$), 4.17-4.15 (m, 2H, $4_C$, $4_A$), 4.06-3.99 (m, 4H, $2_D$, $3_D$, $4_D$, $6_C$), 3.92-3.76 (m, 9H, $3_A$, $6_A$, $5_A$, $4_B$, $6_C$, $5_D$, OC$H_3$), 3.72-3.59 (m, 6H, $6_A$, $3_C$, $6_D$, $6_D$, $2_C$, $5_C$), 2.02 (s, 3H, NHCOC$H_3$), 2.01 (s, 3H, NHCOC$H_3$), 1.45 (s, 3H, pyruvate C$H_3$), 1.30 (d, *J* = 6.6 Hz, 3H, $6_B$)

$^{13}$C NMR: (150 MHz, $CDCl_3$)

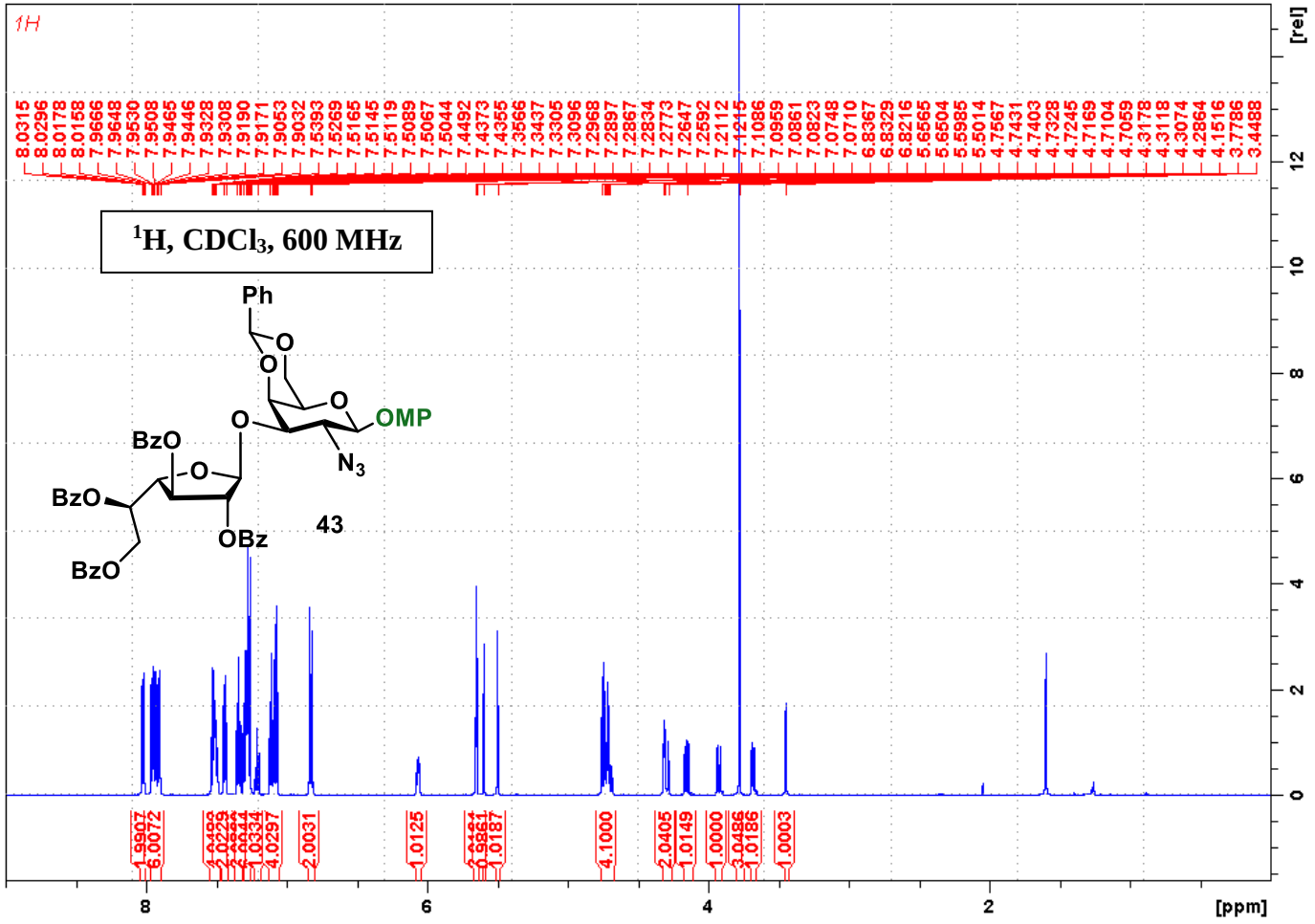
δ 175.8 (*C*OOH), 174.8 (NH*C*O$CH_3$), 174.6 (NH*C*O$CH_3$), 154.8 (Ar-*C*), 150.7 (Ar-*C*), 118.3 (Ar-*C*), 114.9 (Ar-*C*), 109.1 ($1_D$), 104.2 ($1_C$), 100.9

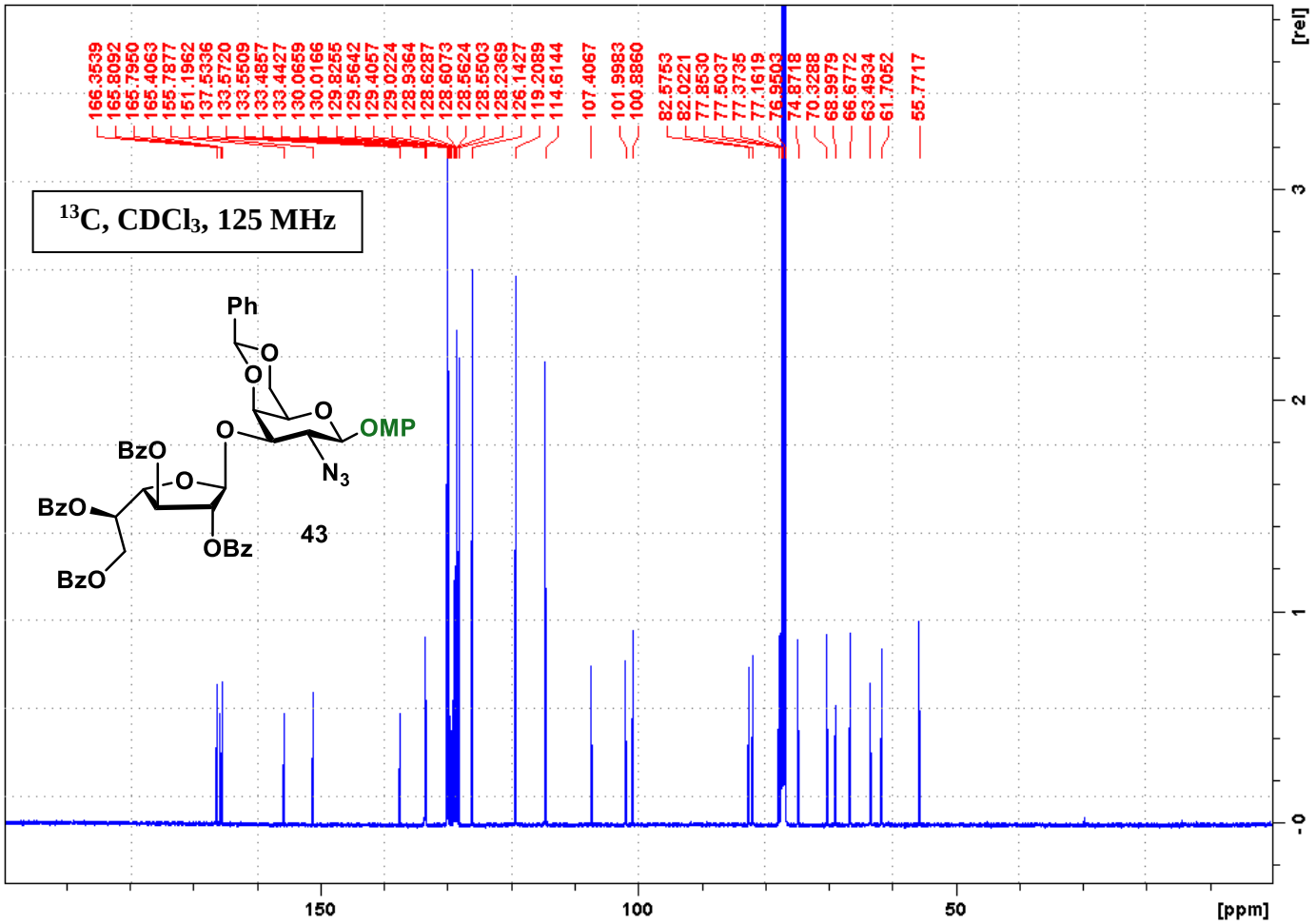
(pyruvate quaternary carbon), 100.7 ($1_A$), 98.1 ($1_B$), 81.6 ($4_D$), 80.5 ($2_D$), 77.9 ($3_A$, $4_B$), 76.2 ($4_A$), 75.5 ($5_A$), 75.3 ($3_D$), 71.4 ($5_C$, $3_C$), 70.8 ($4_C$), 70.2 ($5_D$), 69.9 ($2_C$), 66.3, 65.1 ($6_C$), 62.6 ($6_D$), 59.9 ($6_A$), 55.7 (OC$H_3$), 51.6 ($2_A$, $3_B$), 48.7 ($2_B$), 24.9 (pyruvate C$H_3$), 22.0 (NHCOC$H_3$), 21.8 (NHCOC$H_3$), 16.2 ($CH_3/6_B$)

HRMS: Exact mass calcd. for $C_{38}H_{57}N_3O_{22}^+$ $[M+H]^+$: 908.3512, found 908.3480

OR: $[\alpha]_D^{22.5}$ = +40.0 (C = 0.1 in $D_2O$)

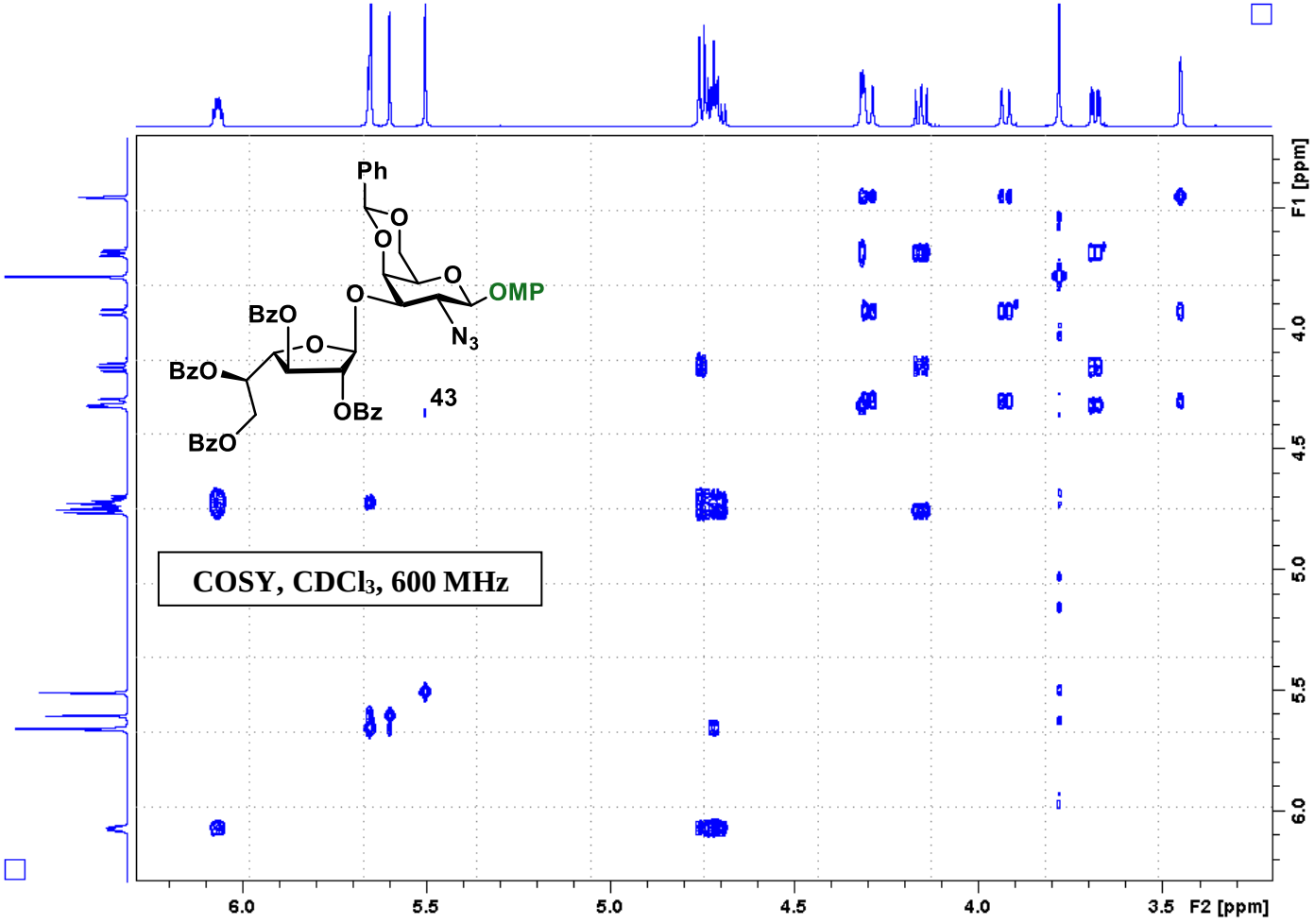
## 2.5.1. Selected NMR spectra:



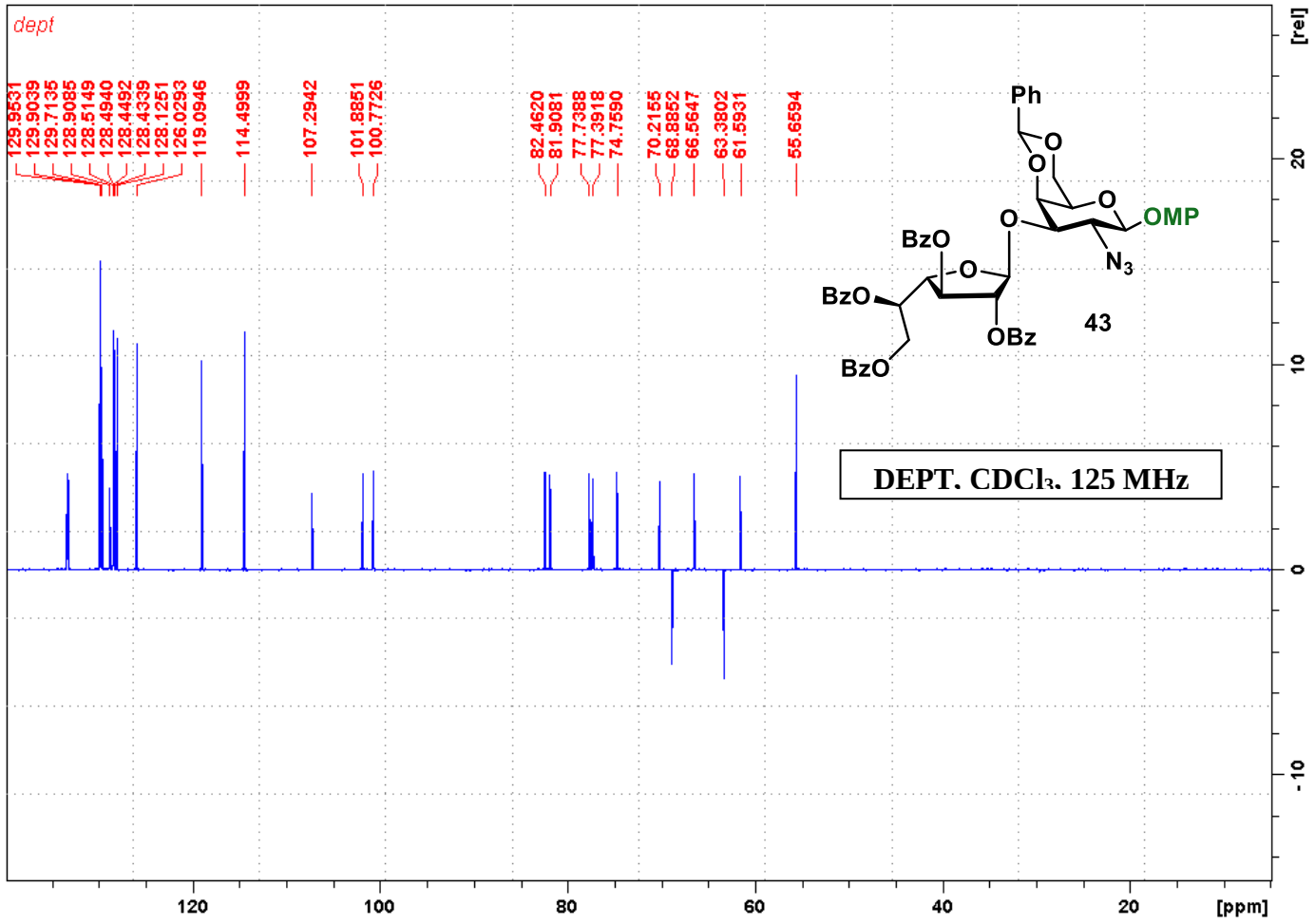



$^{13}$C – Compound **43**

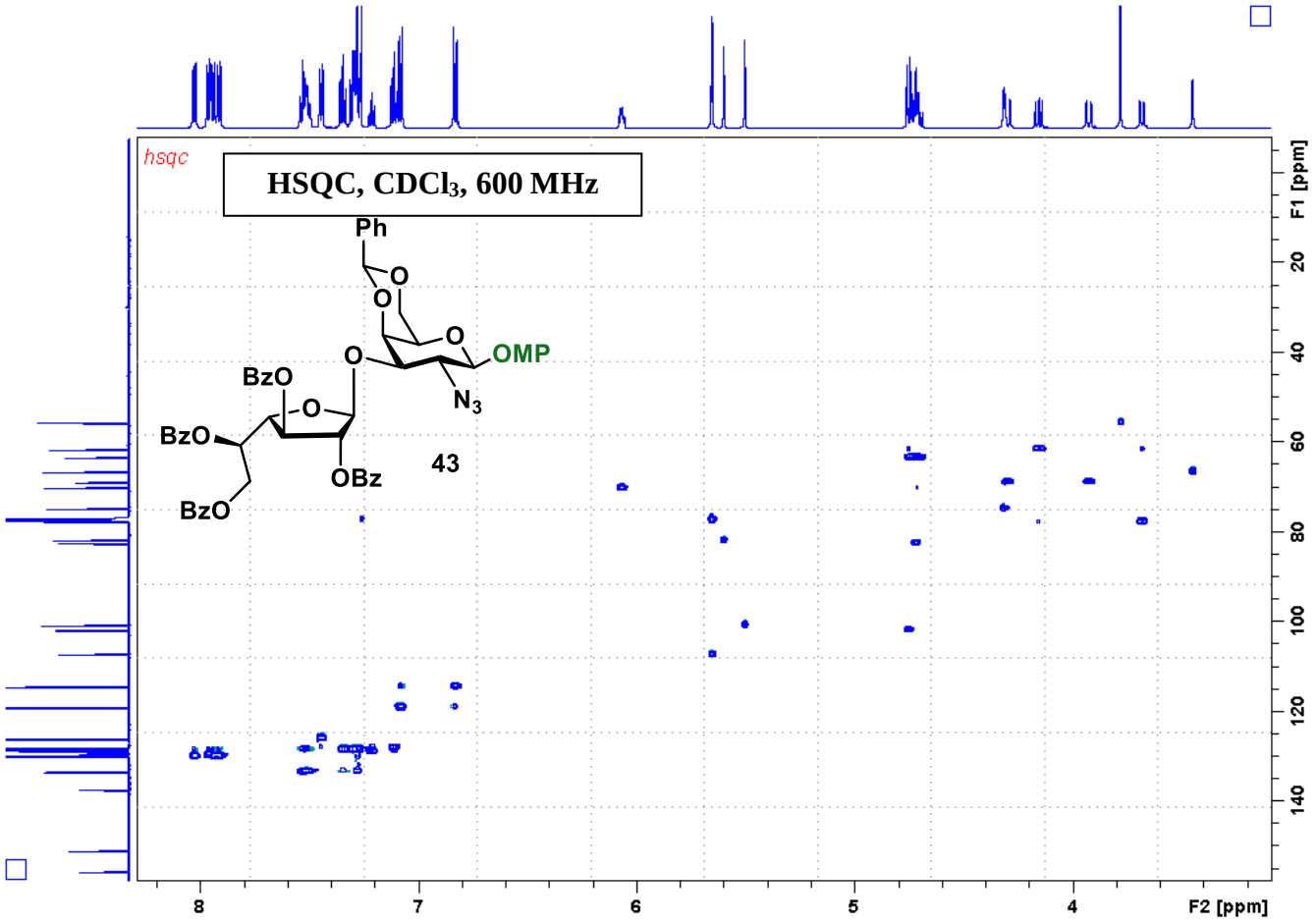
COSY – Compound **43**



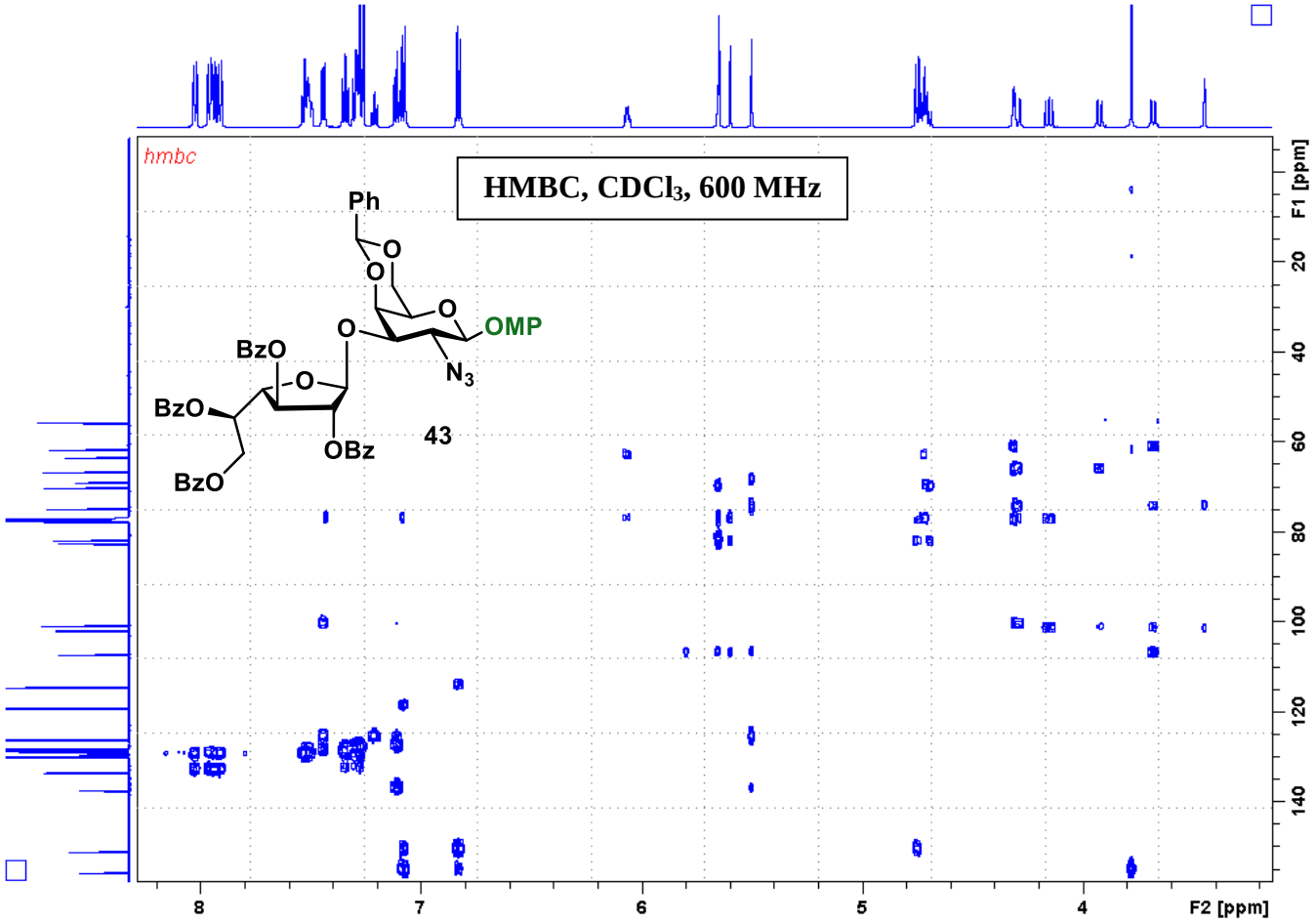
DEPT – compound **43**



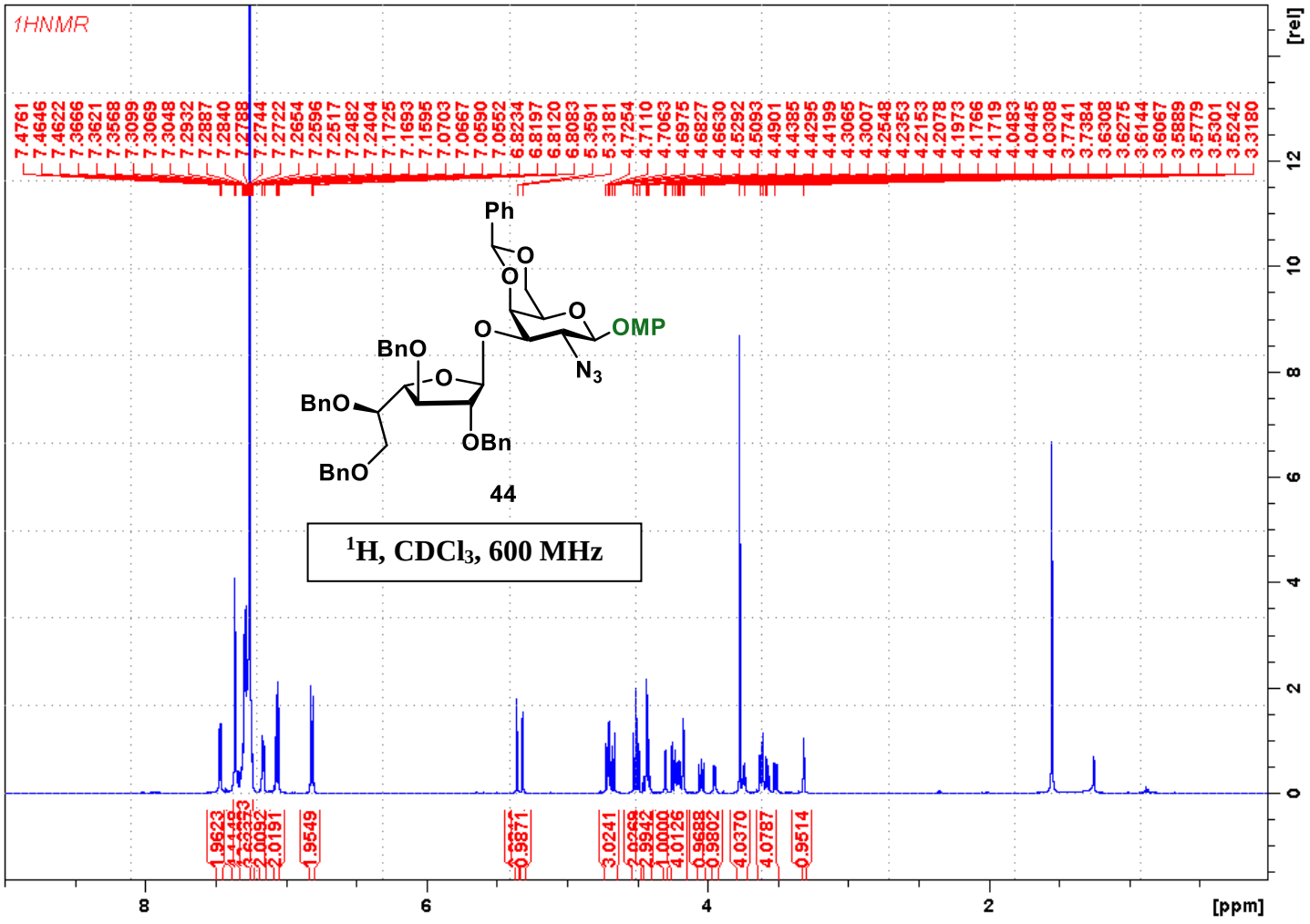
HSQC – Compound **43**



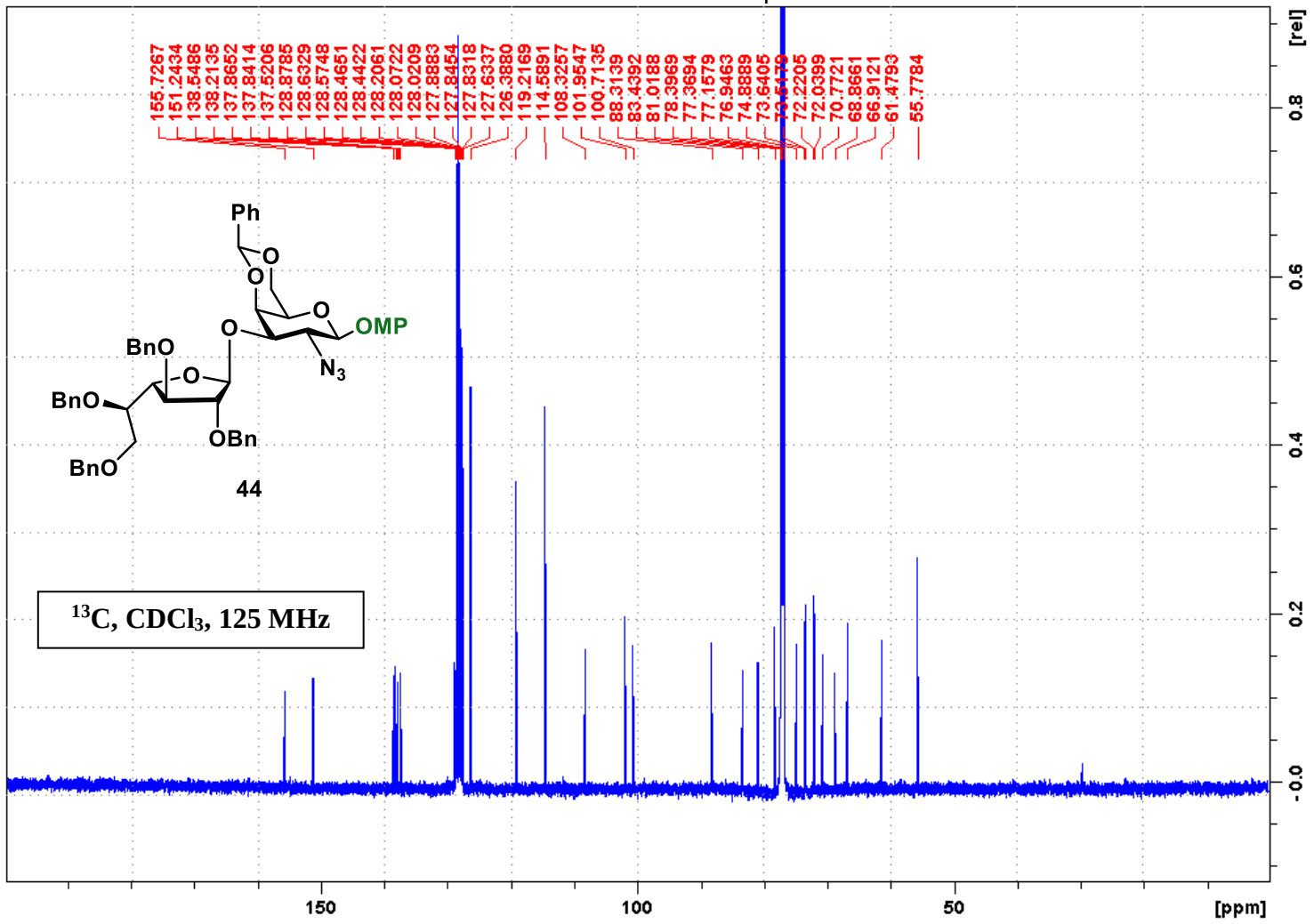
HMBC – Compound **43**



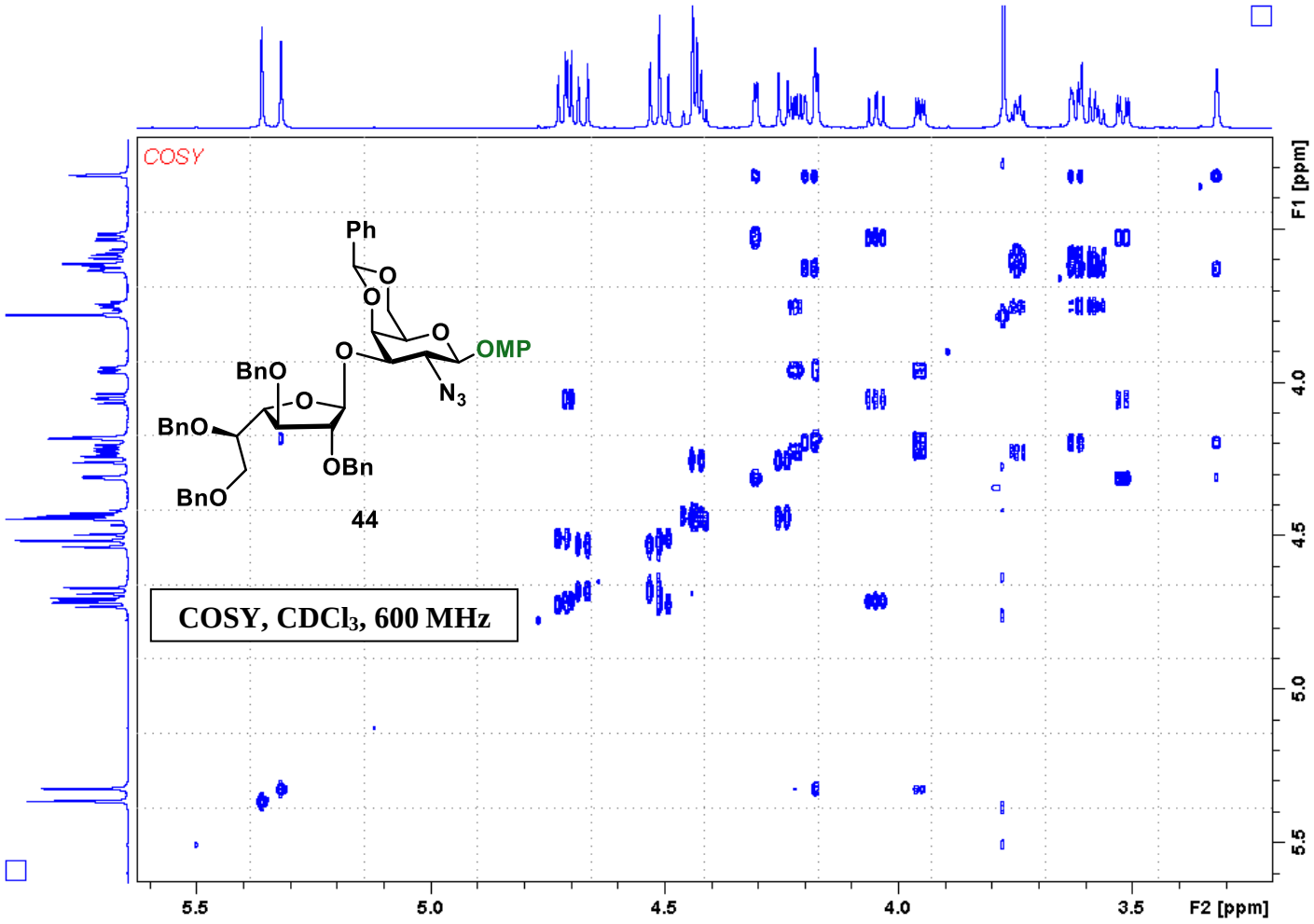
$^{1}$H – Compound **44**



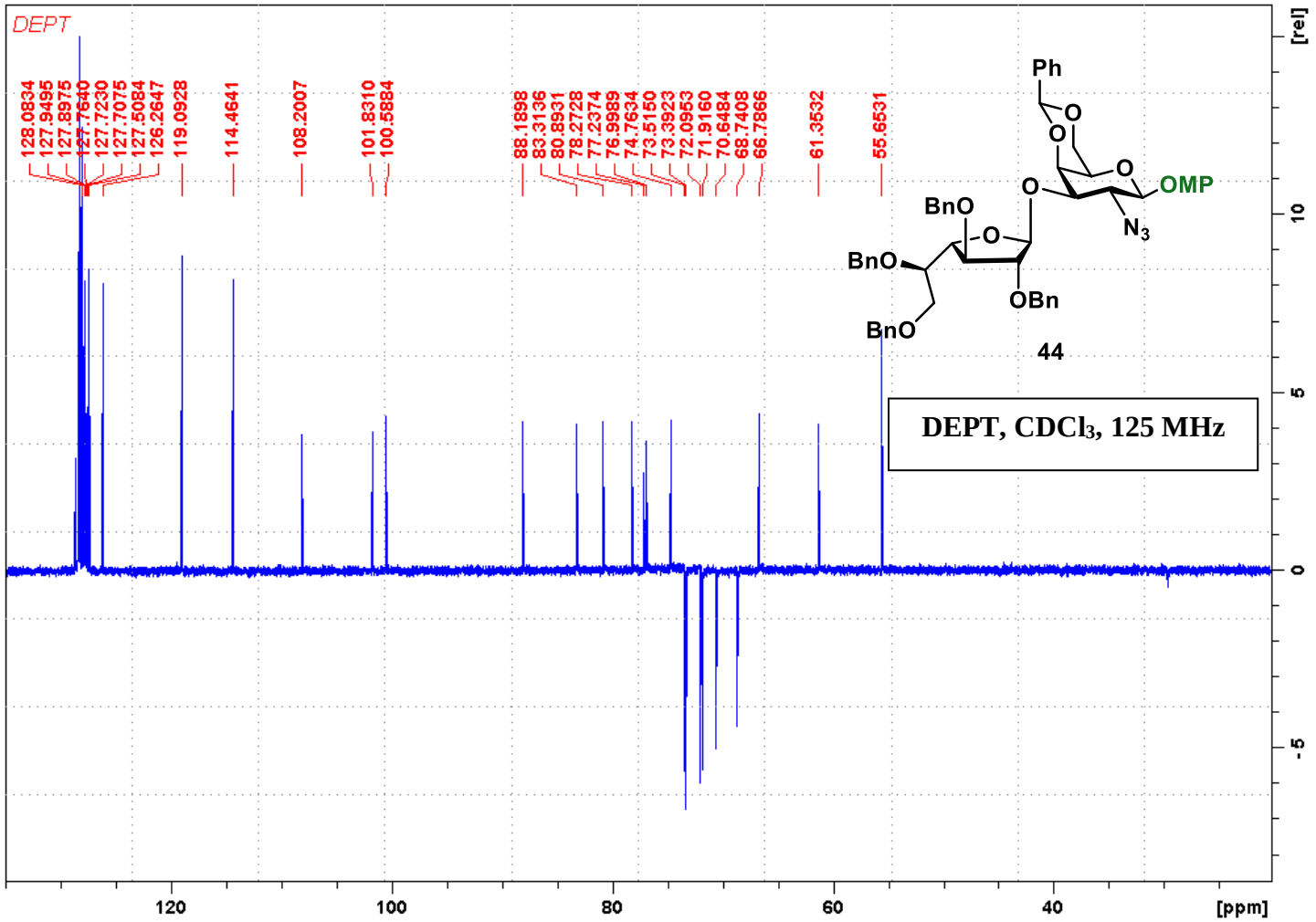
$^{13}$C – Compound **44**



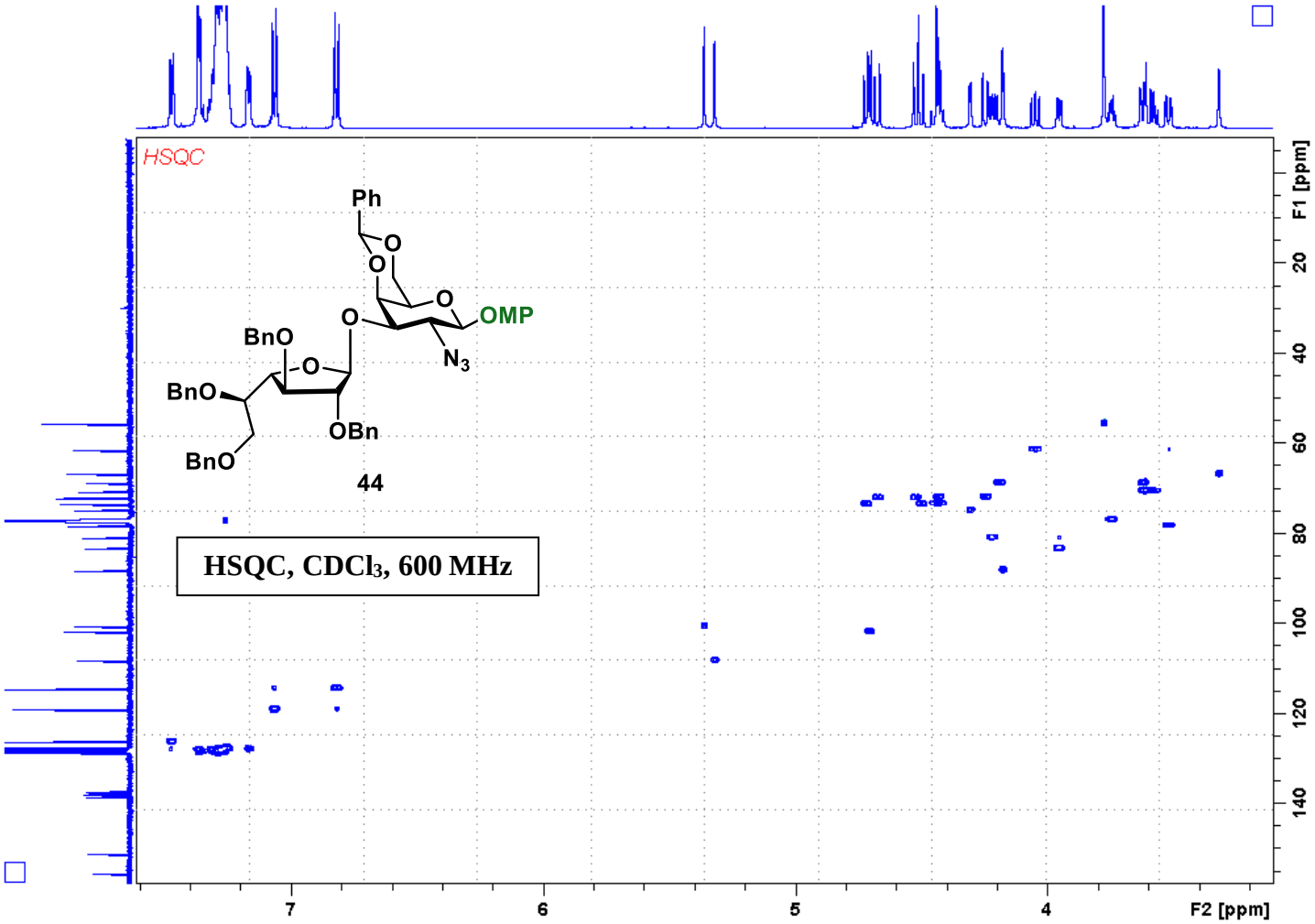
COSY- Compound **44**



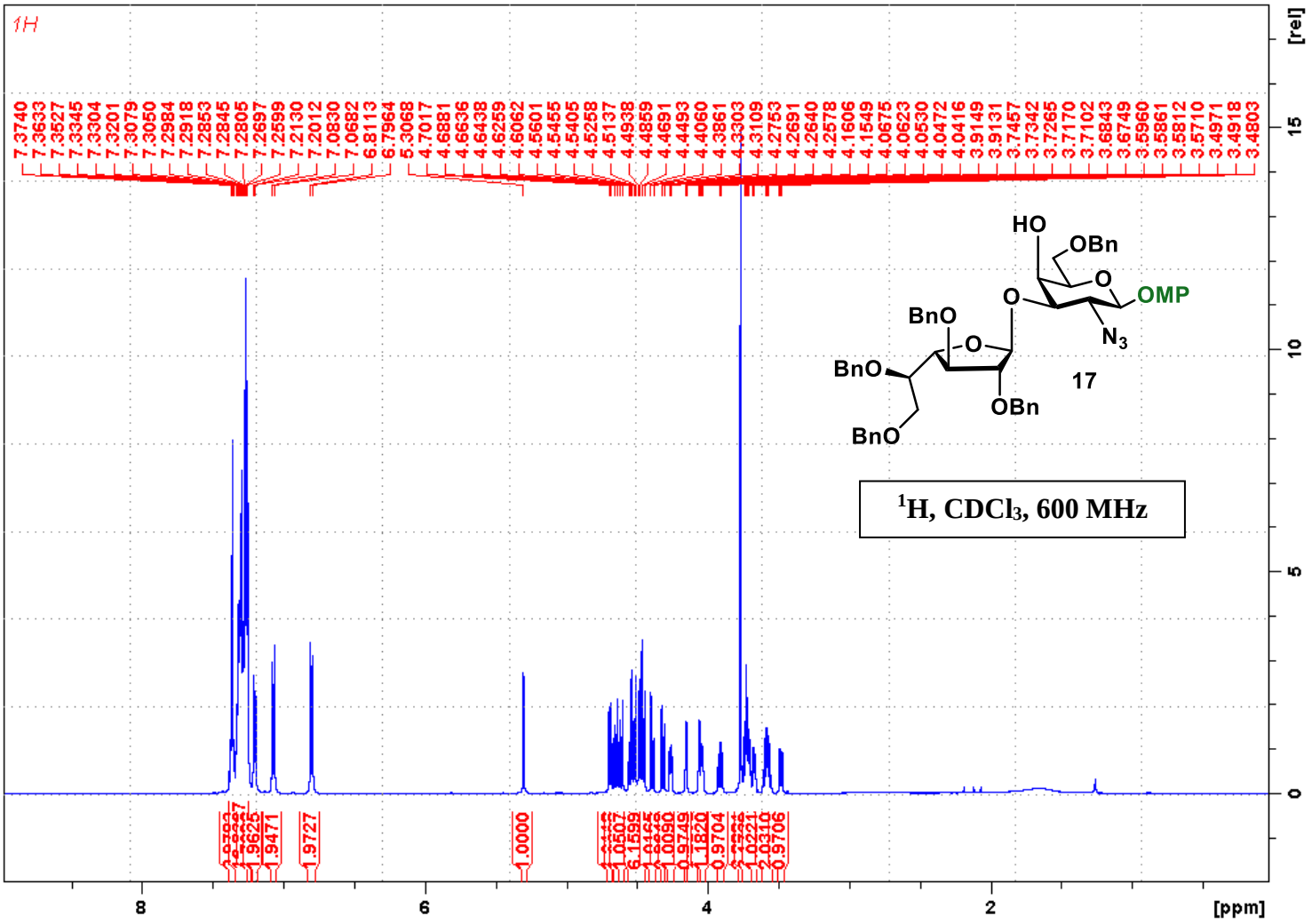
DEPT – Compound **44**



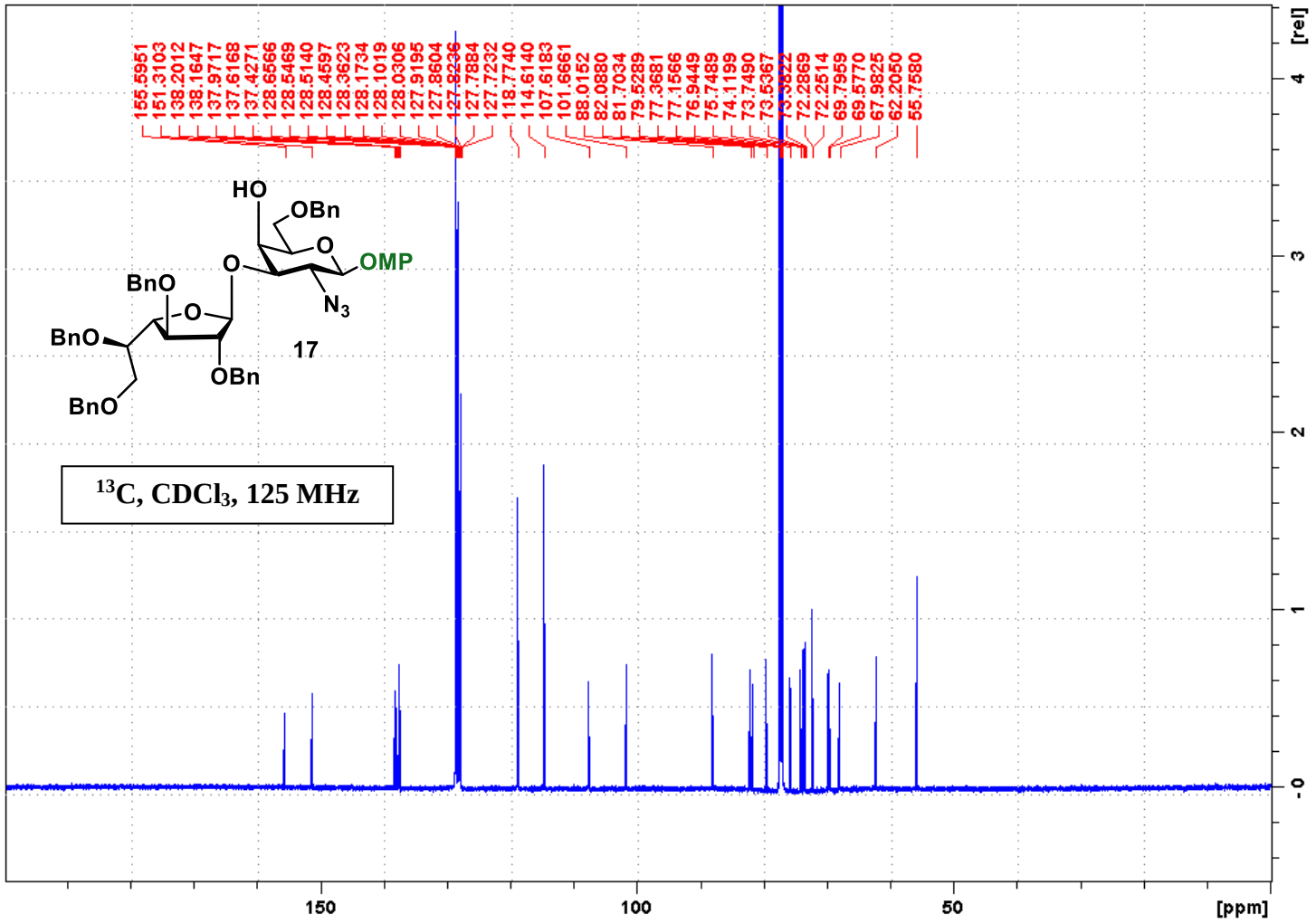
HSQC – Compound **44**



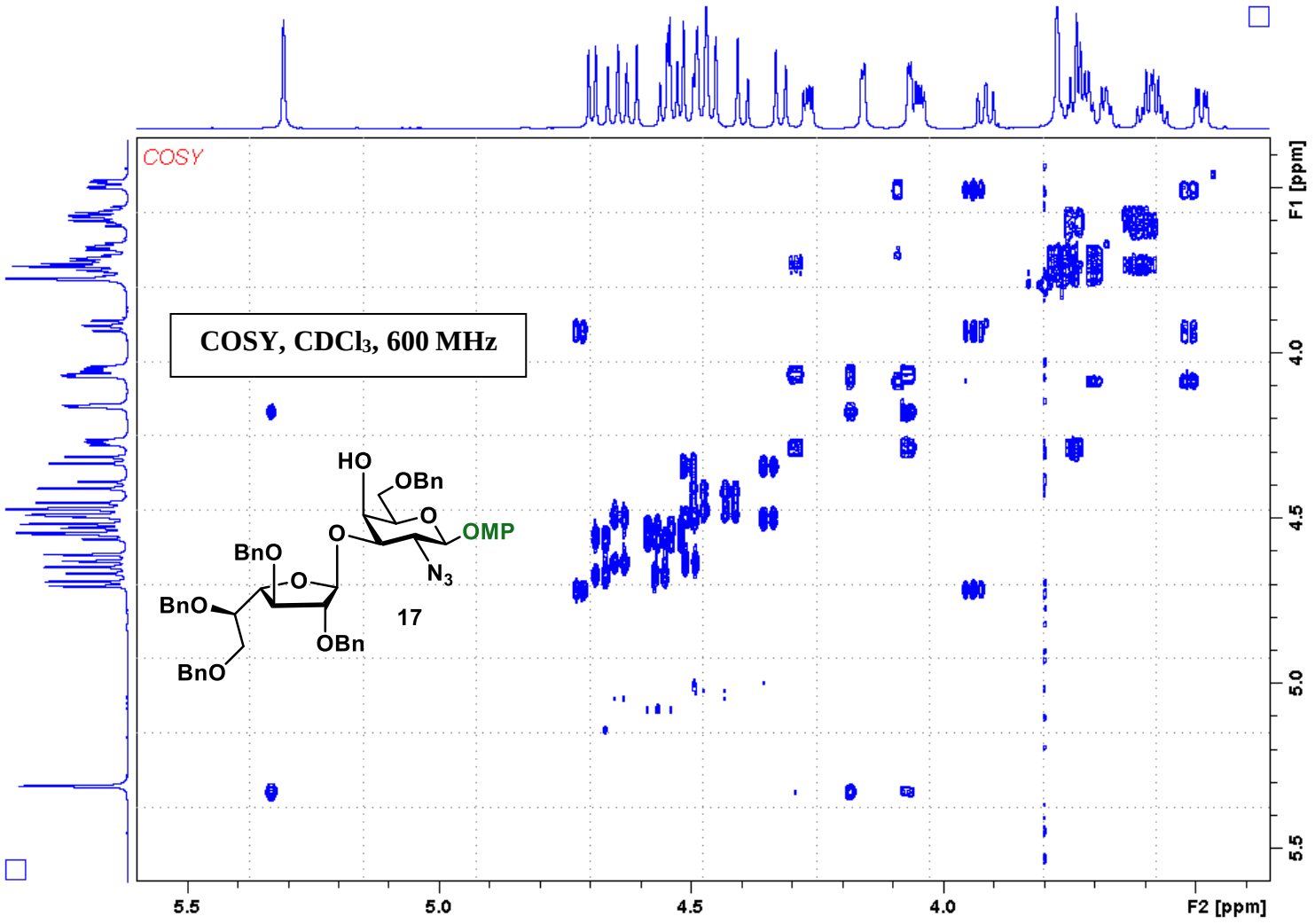
$^{1}$H – Compound **17**



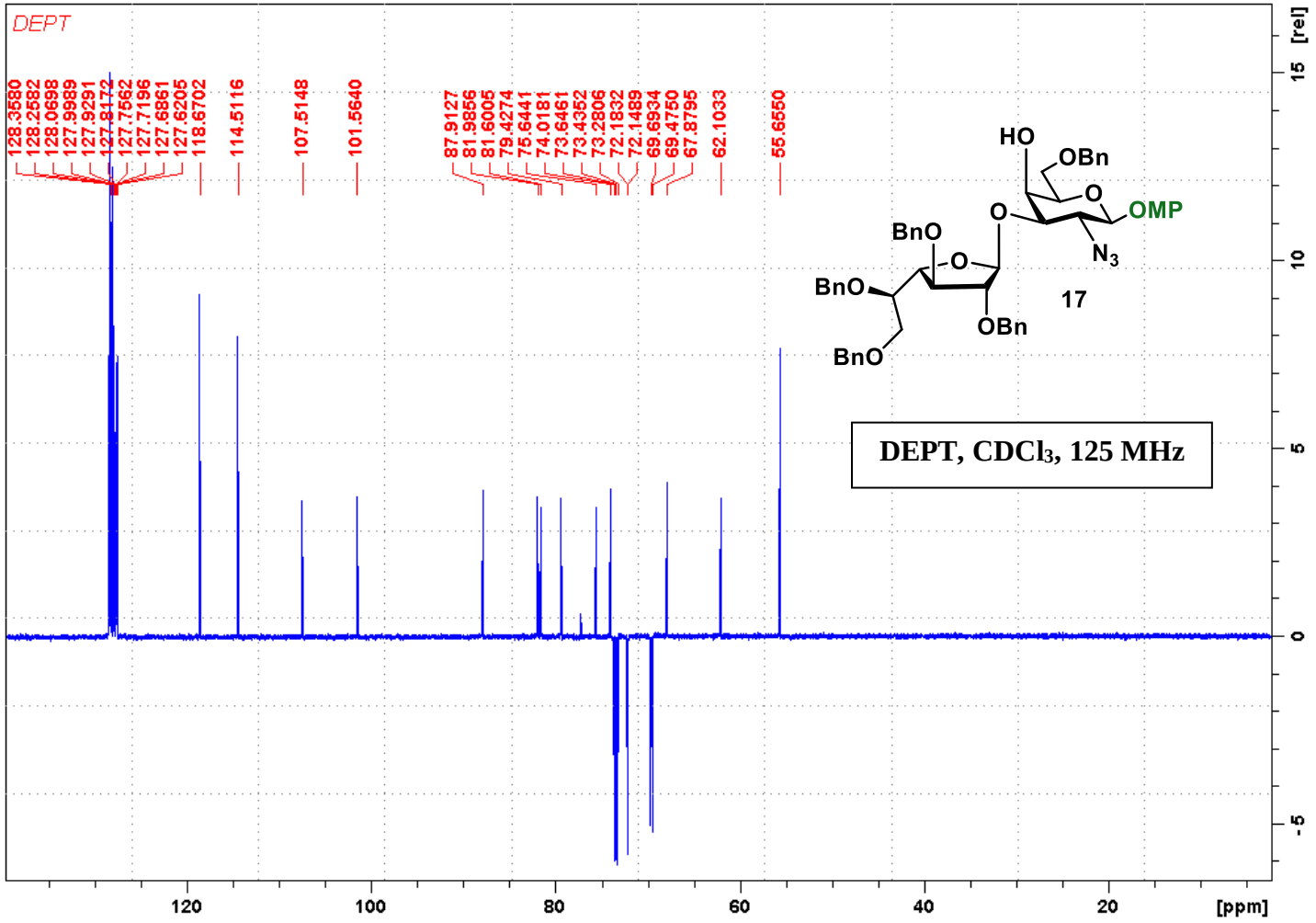
$^{13}C$ – Compound **17**



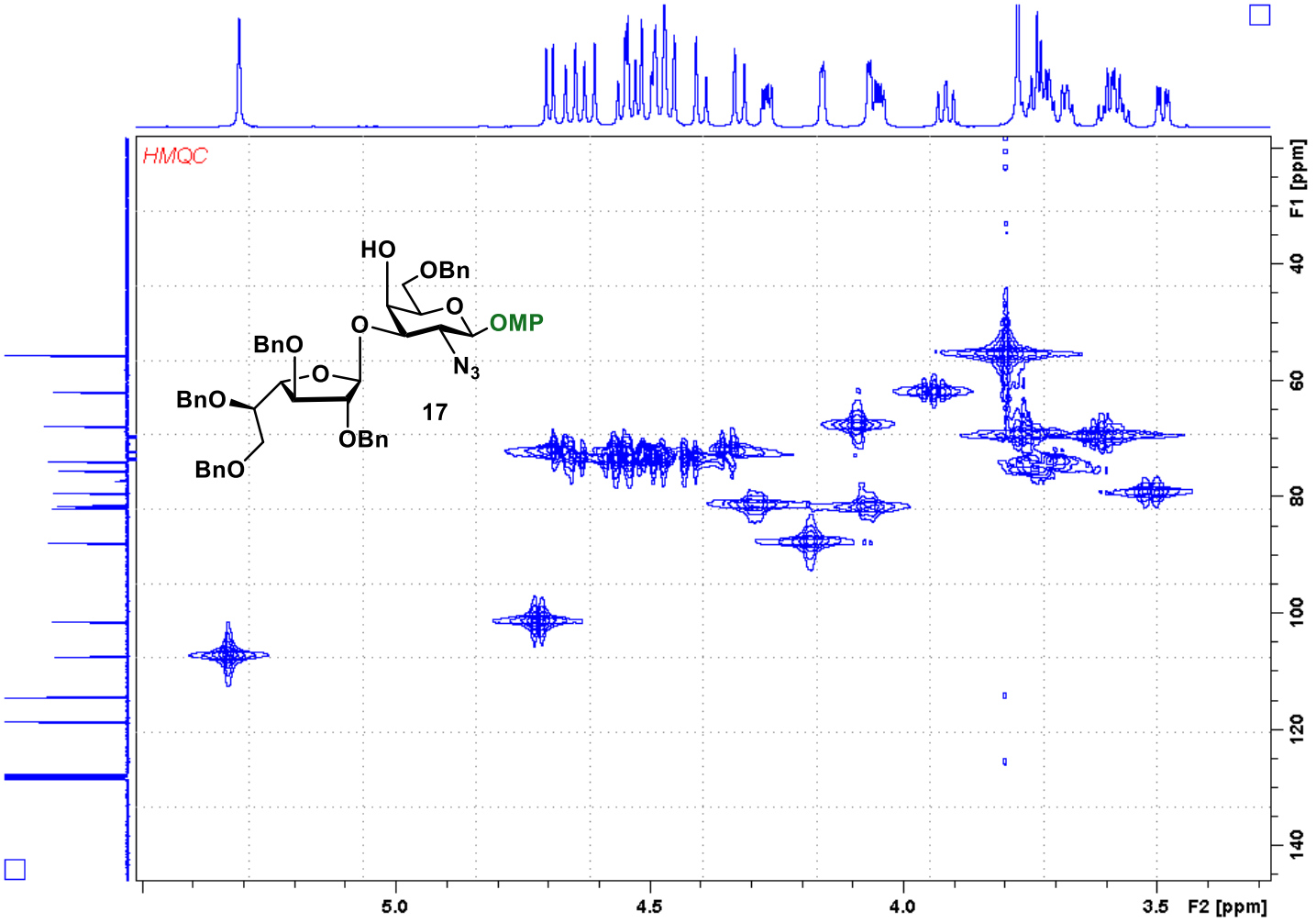
COSY – Compound **17**



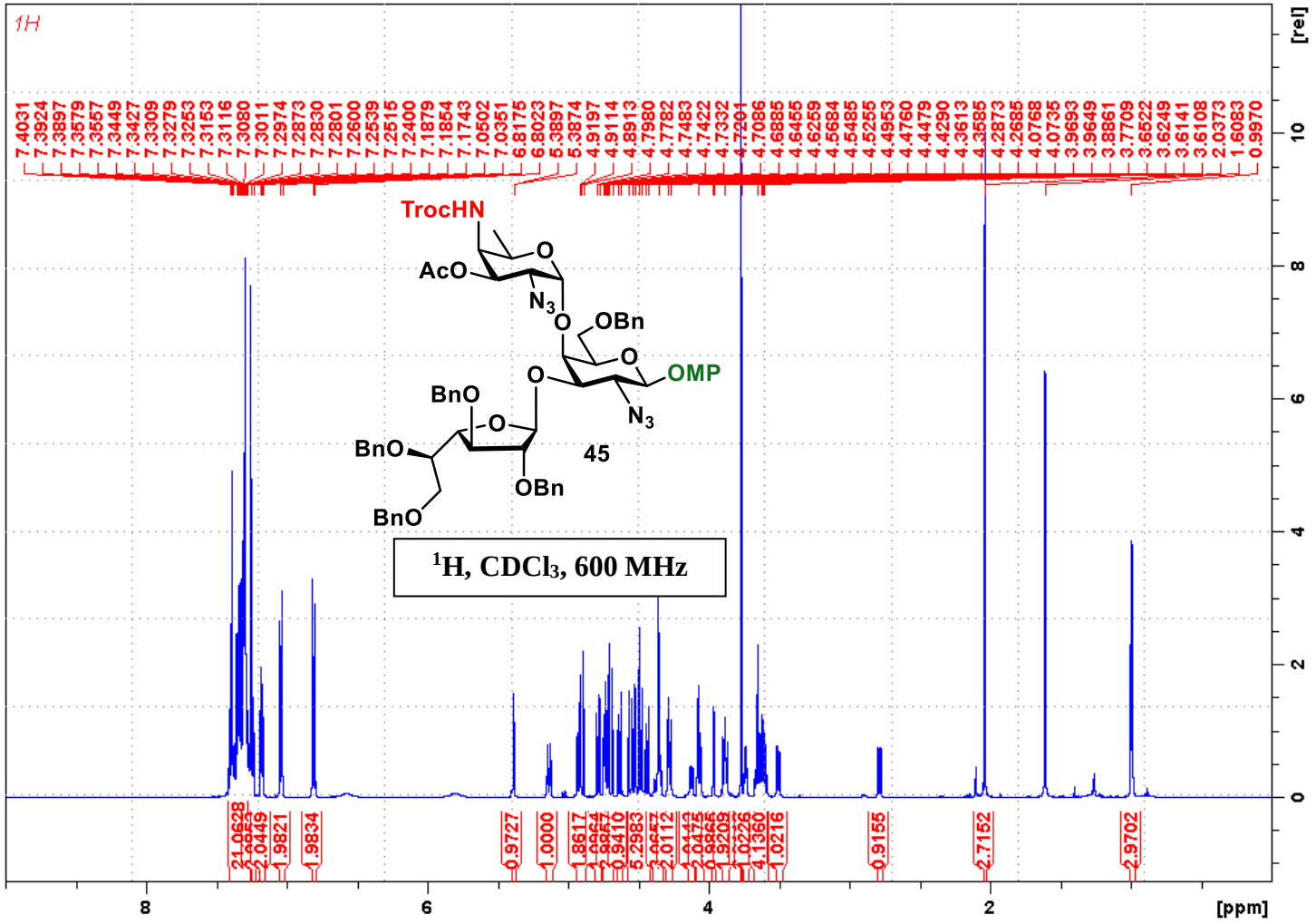
DEPT – Compound **17**



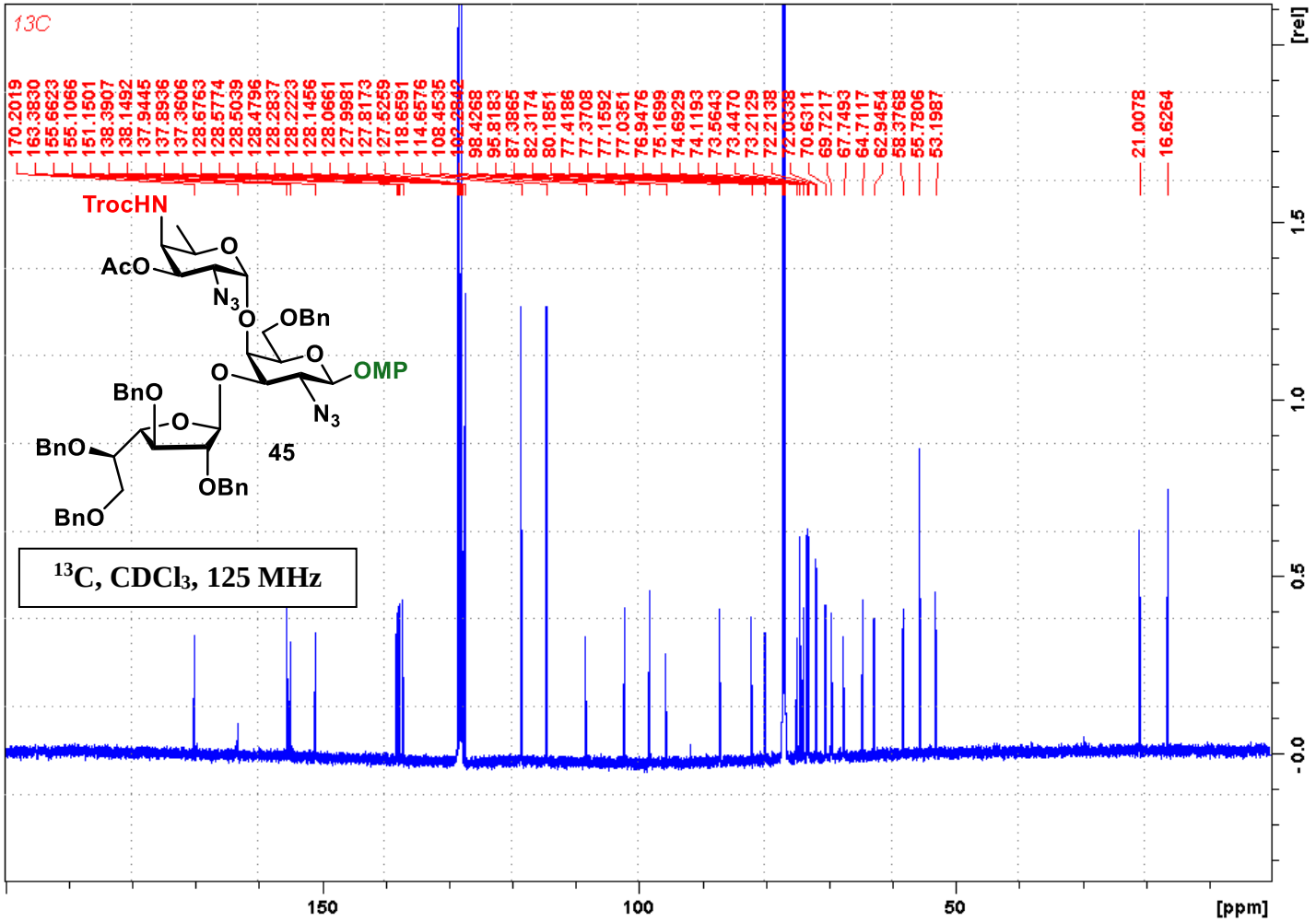
HMQC – Compound **17**



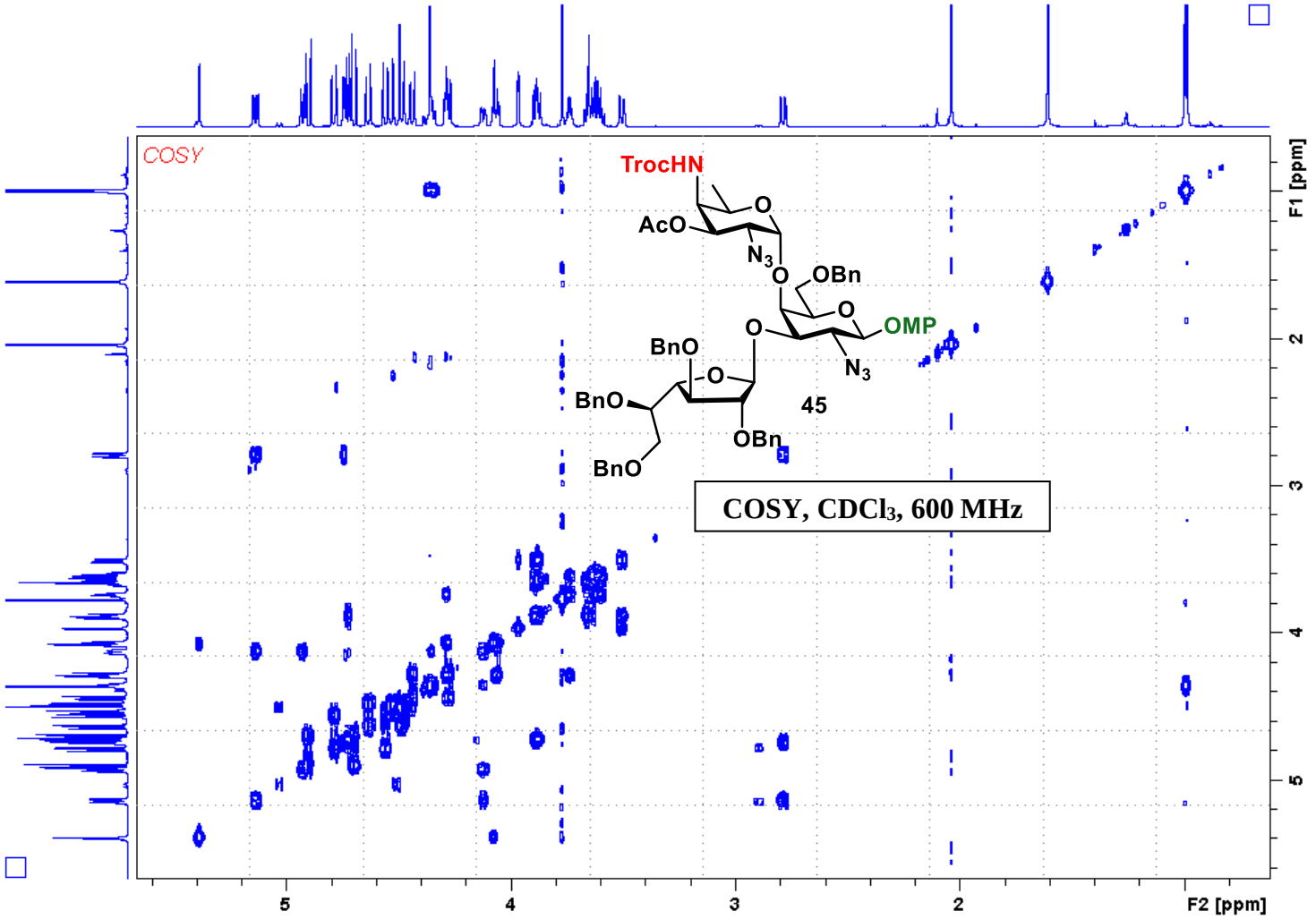
$^{1}$H – Compound **45**



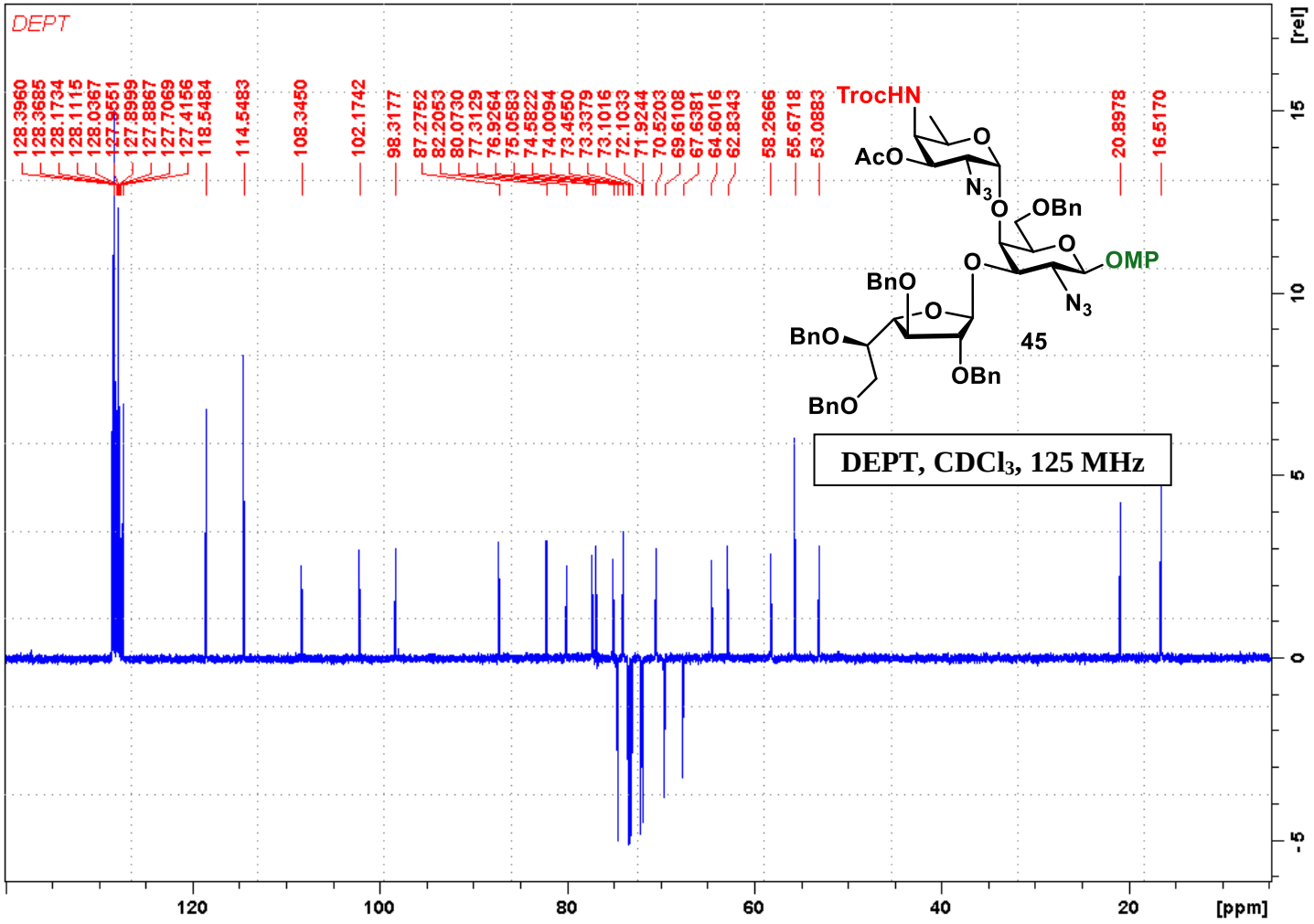
$^{13}C$ – Compound **45**



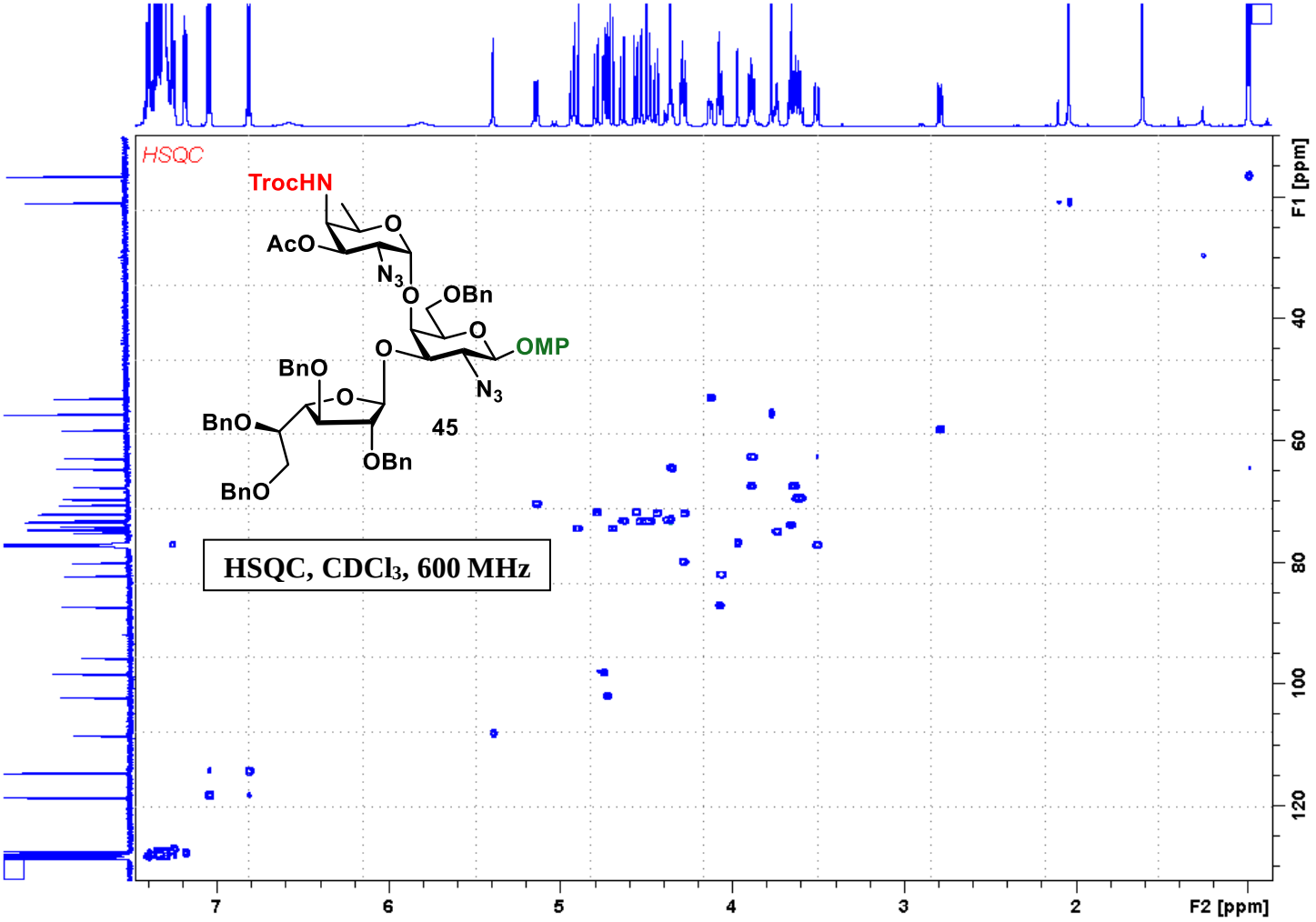
COSY – Compound **45**



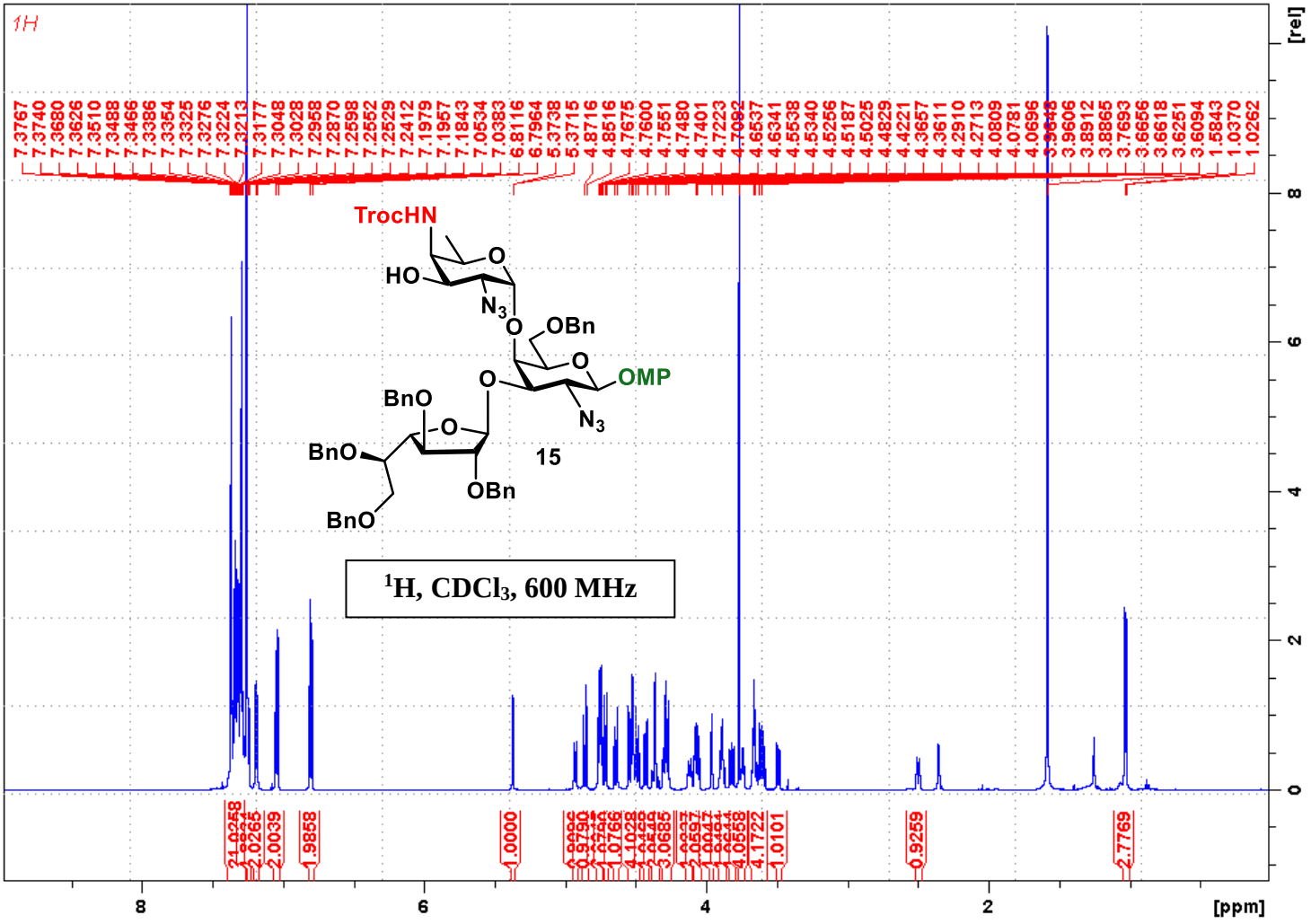
DEPT – Compound **45**



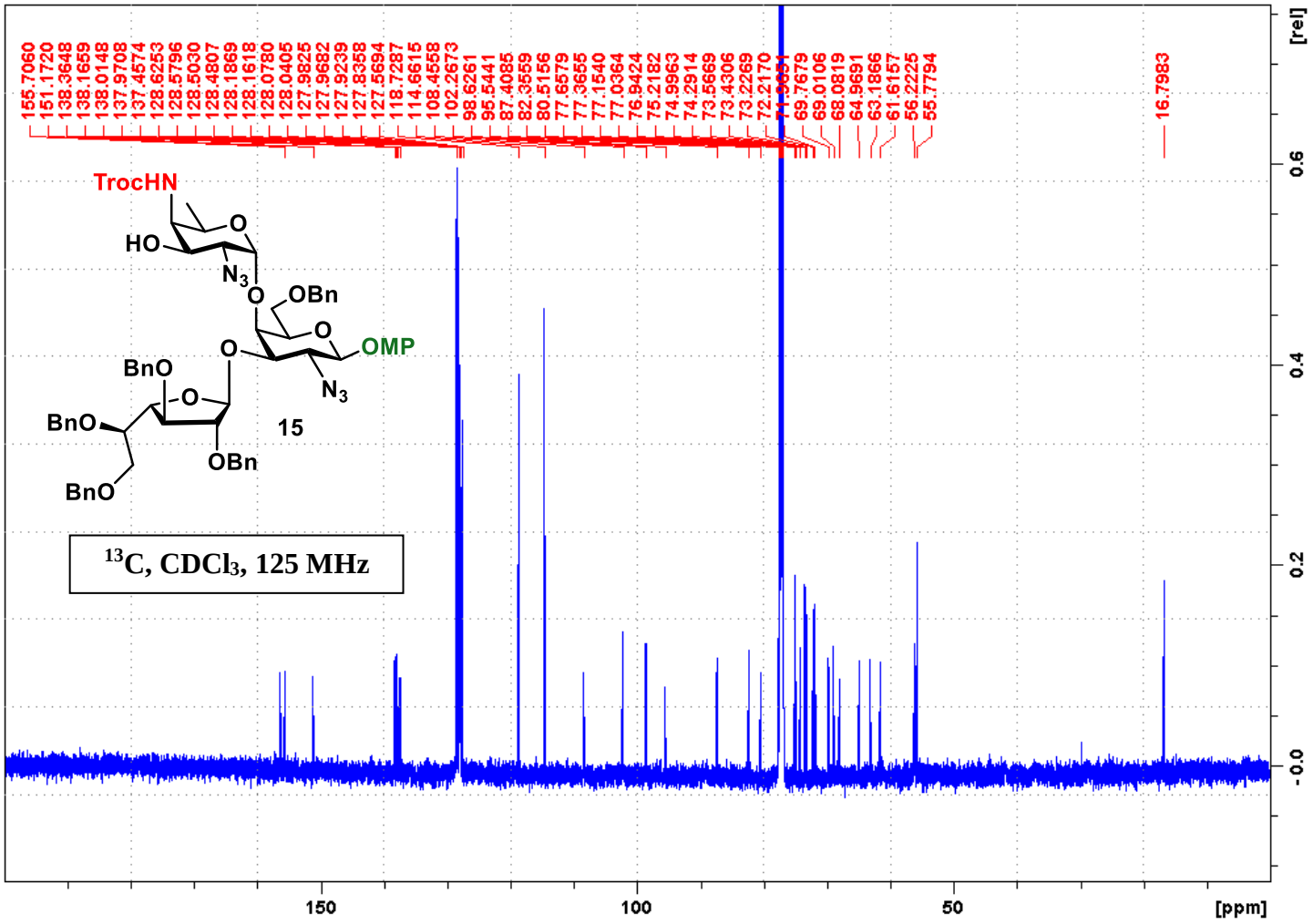
HSQC – Compound **45**



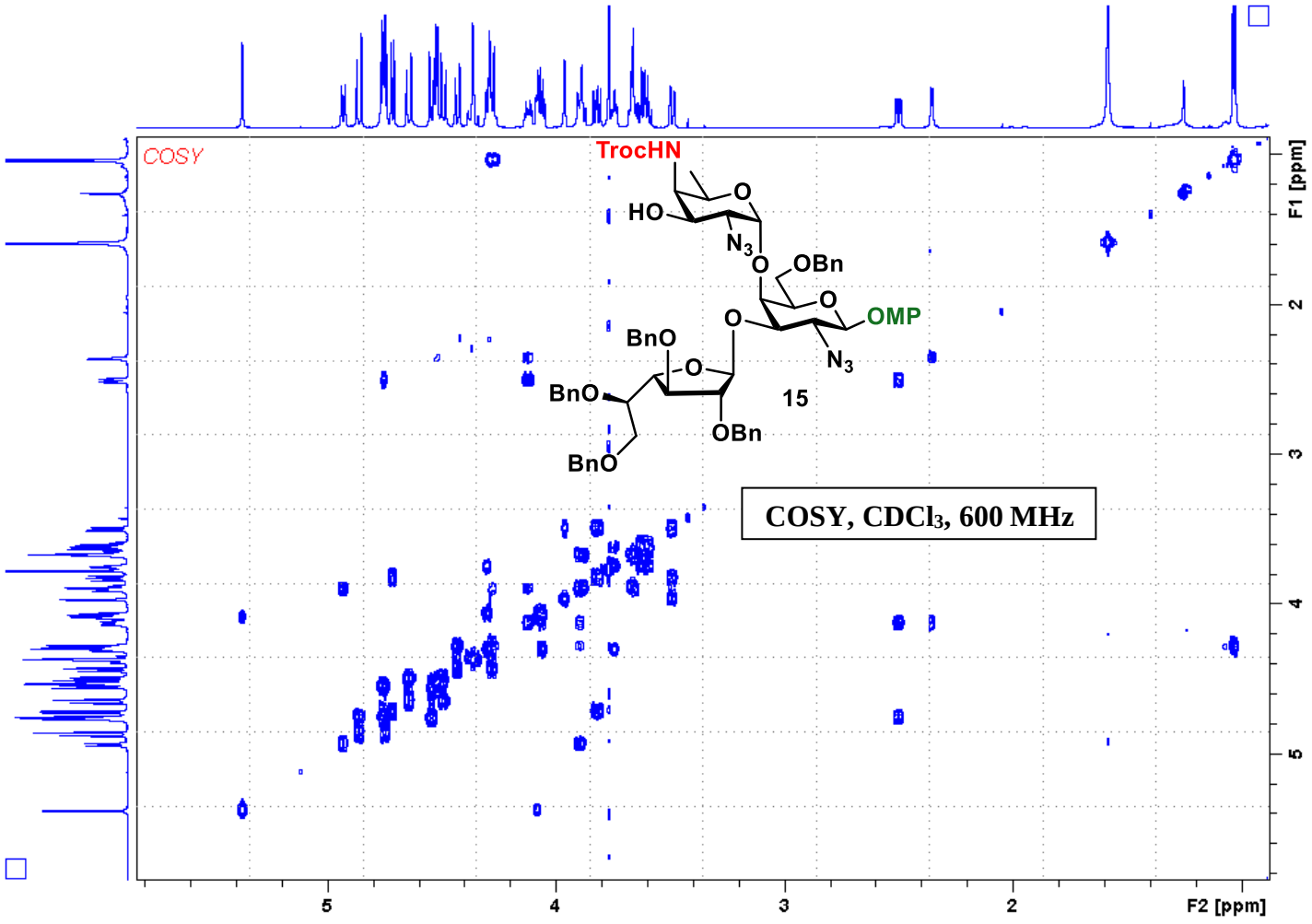
$^{1}$H – Compound **15**



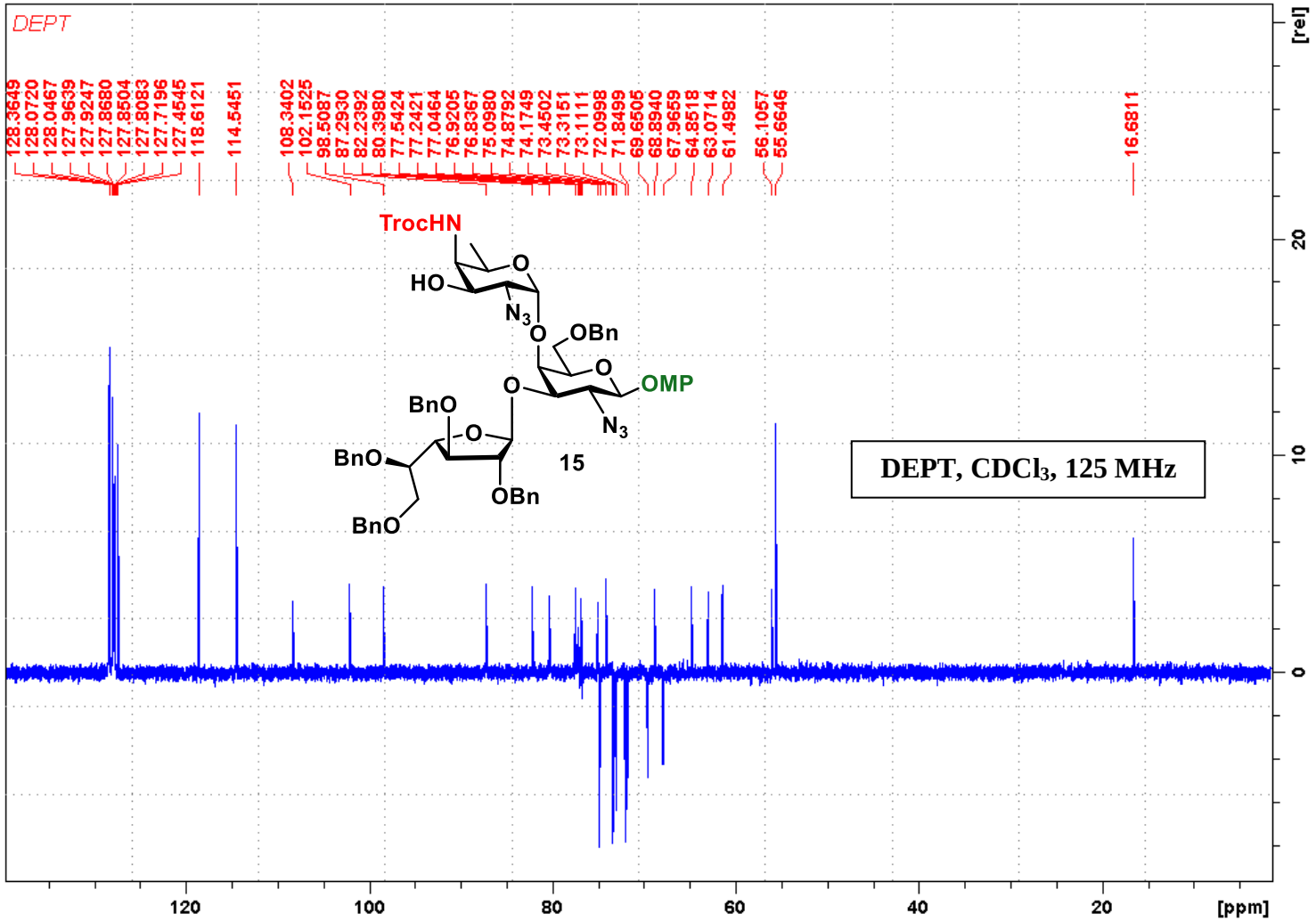
$^{13}$C – Compound **15**



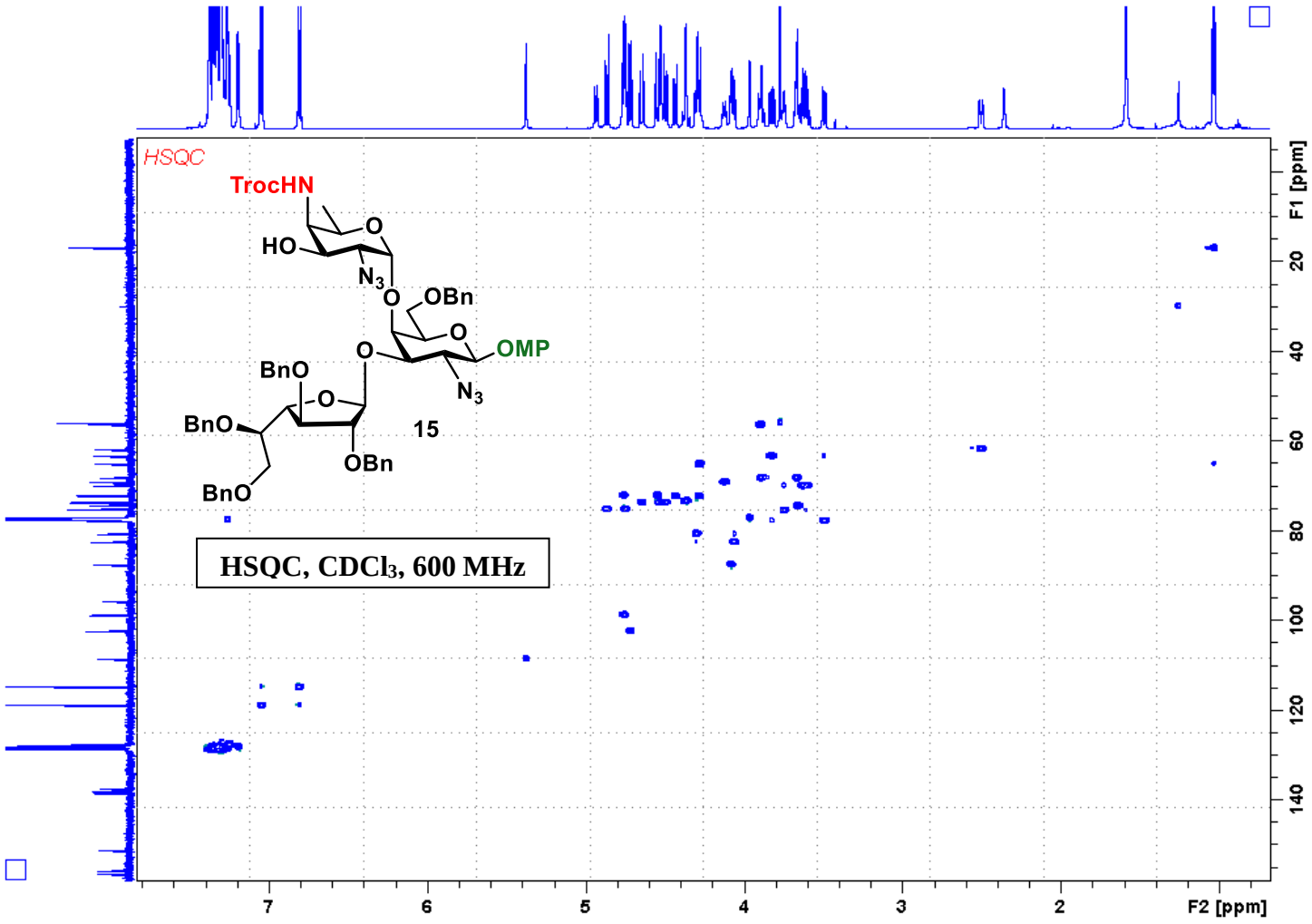
COSY – Compound **15**



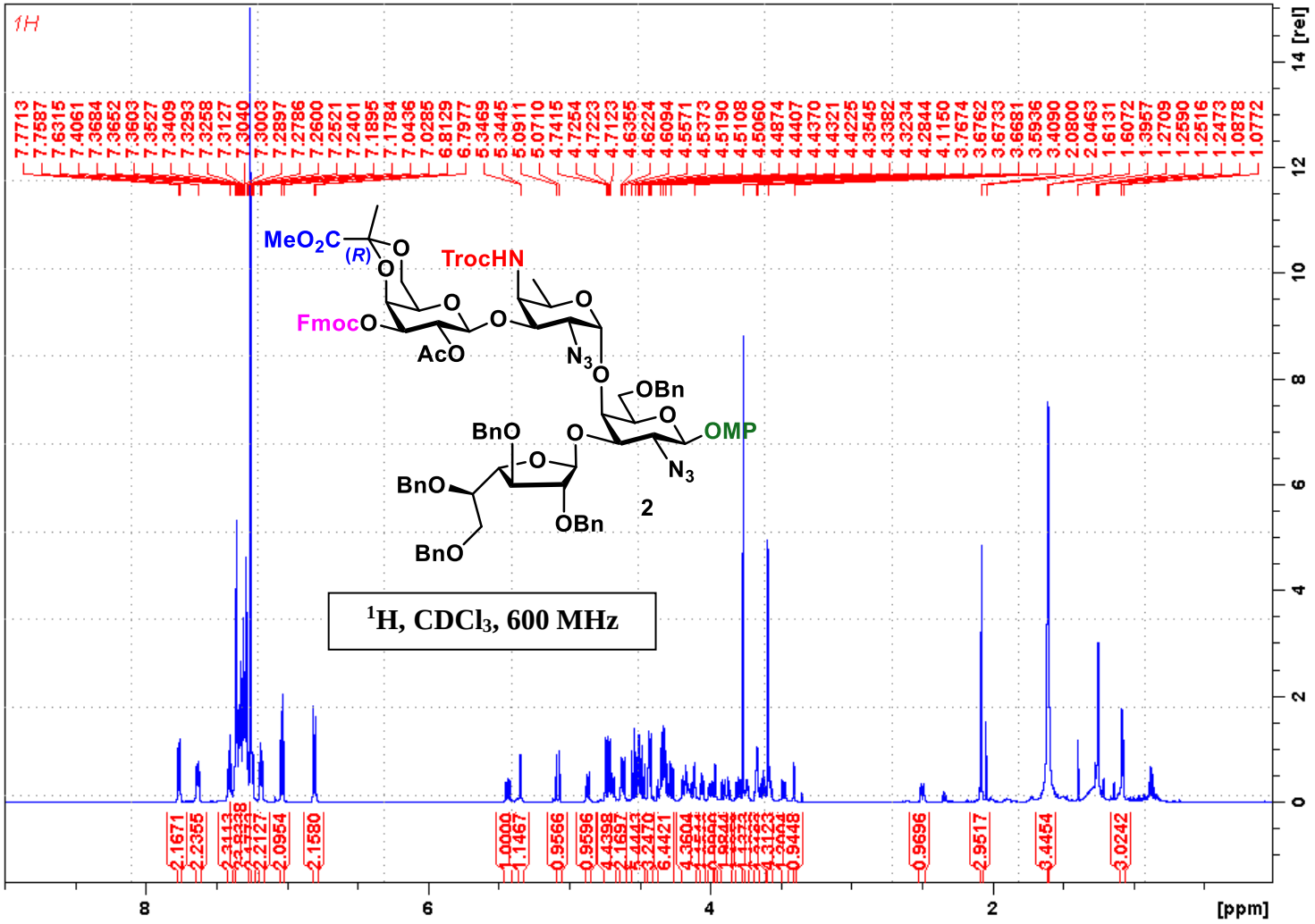
DEPT – Compound **15**



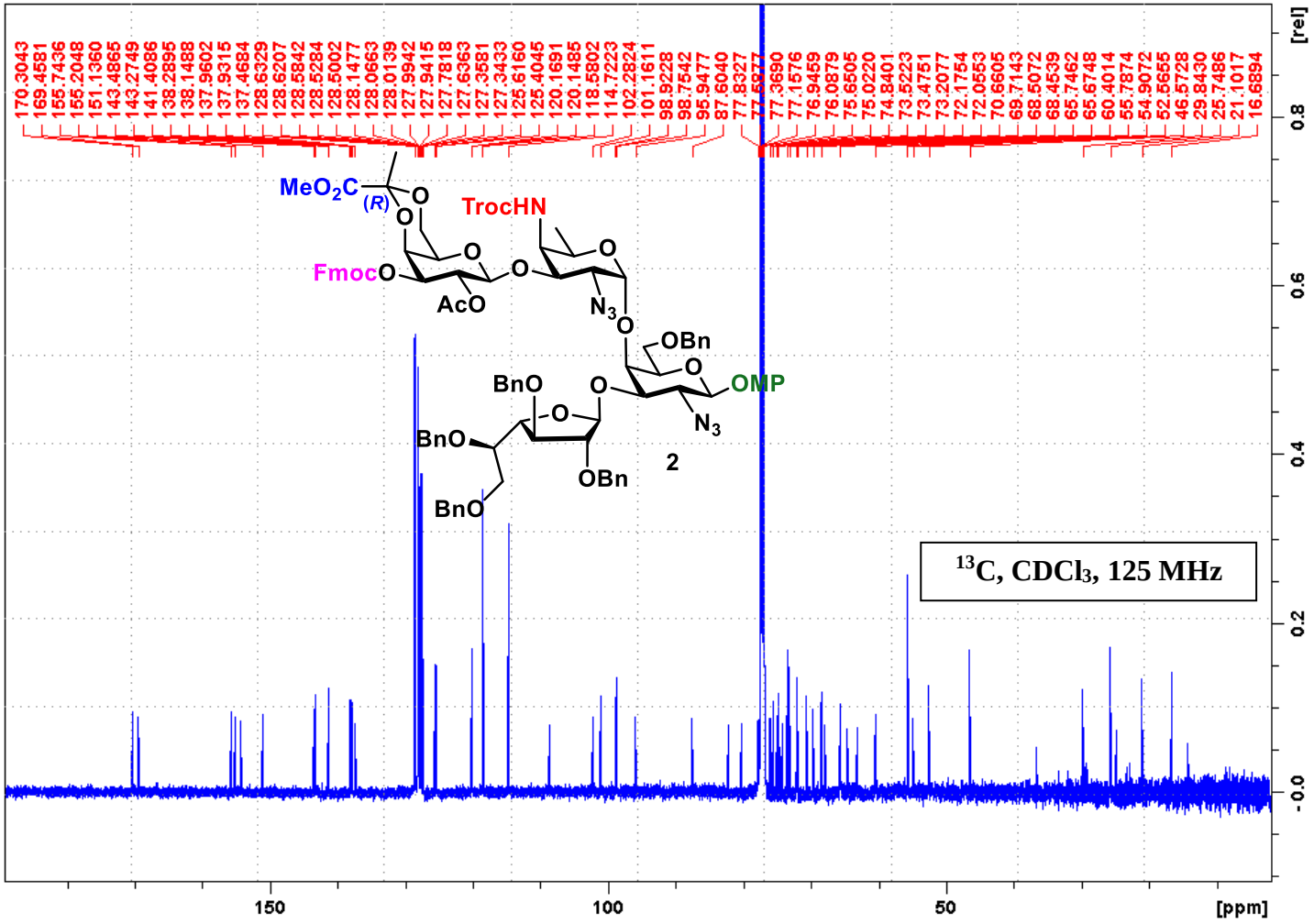
HSQC – Compound **15**



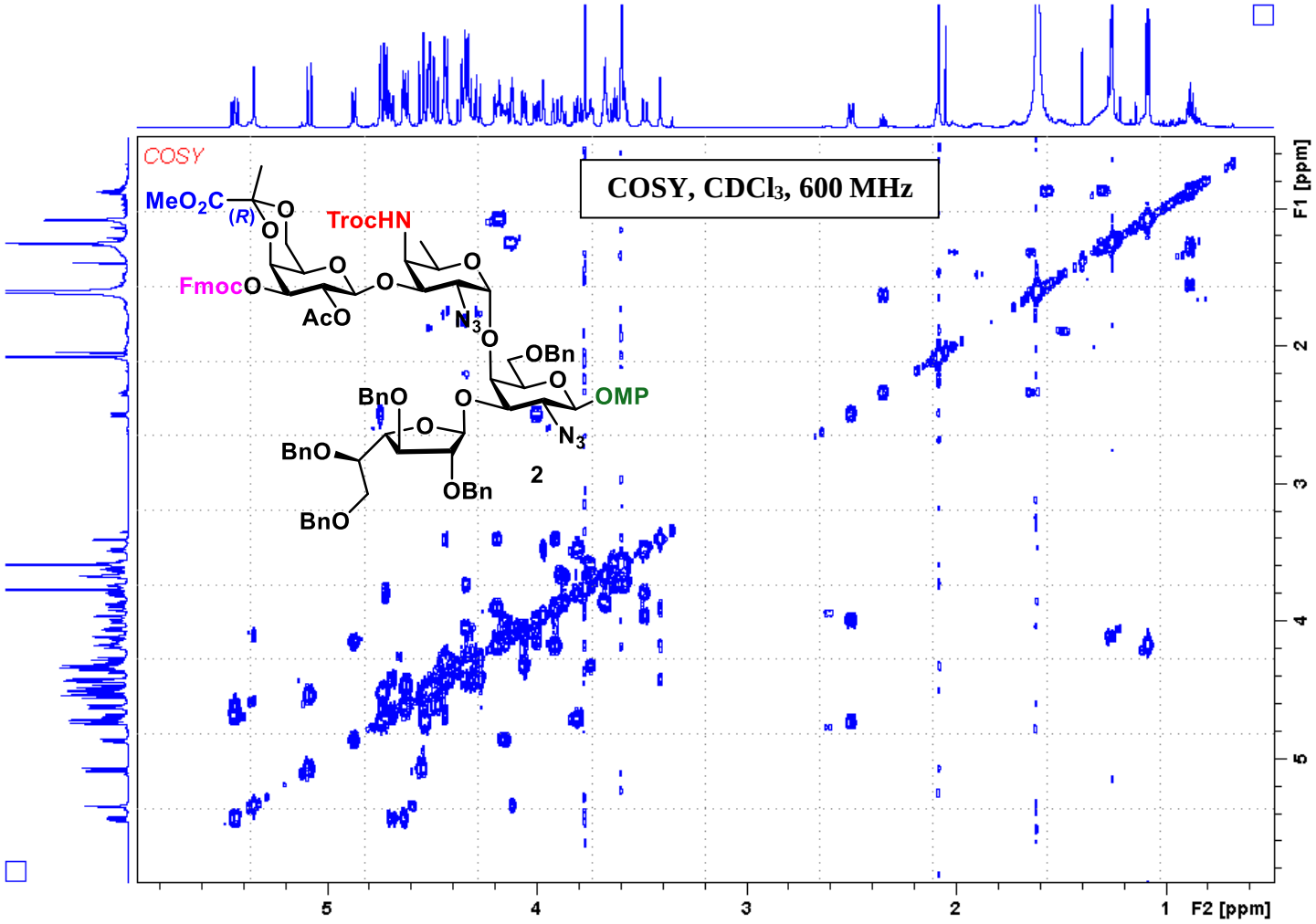
$^{1}$H – Compound **2**



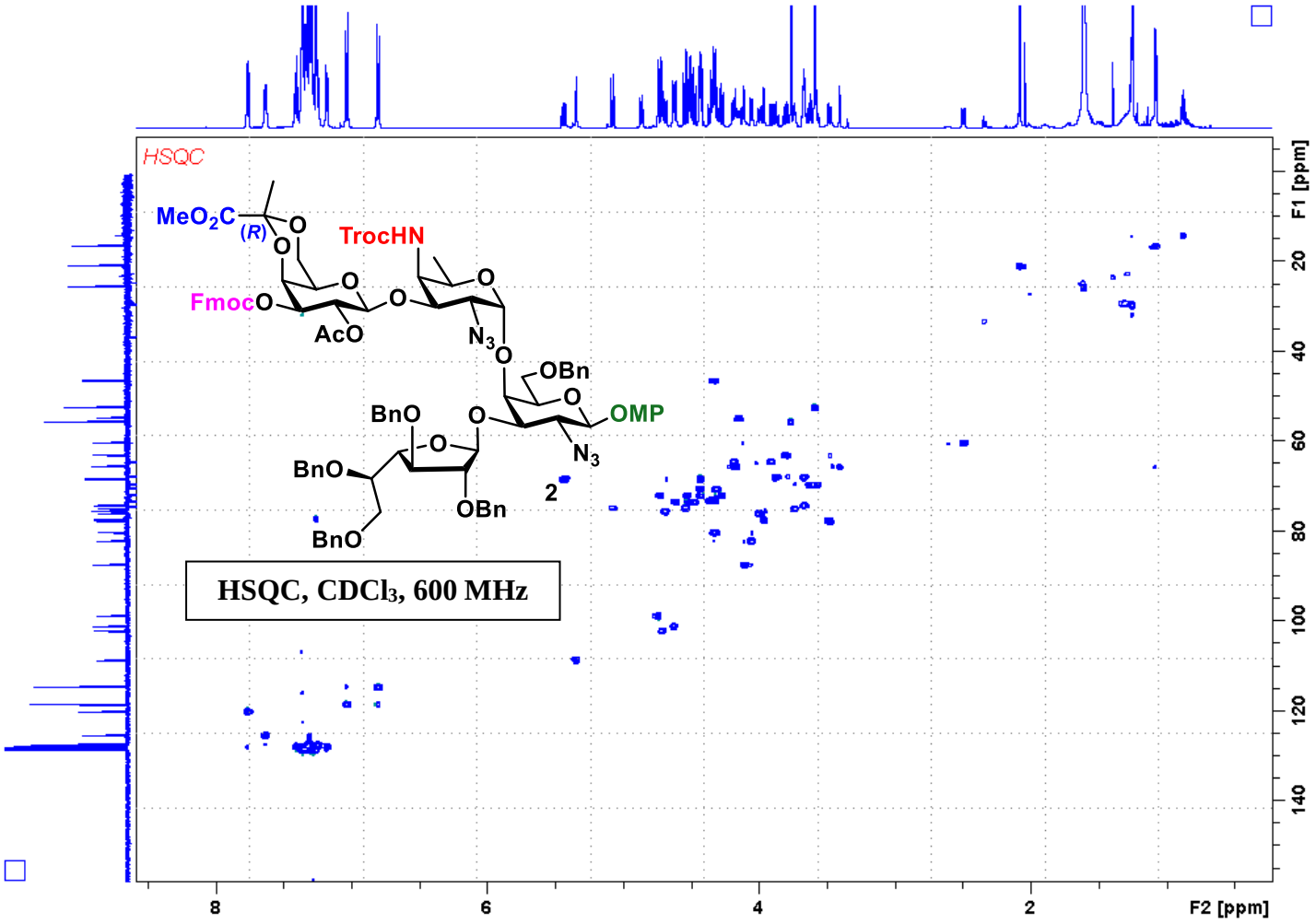
$^{13}$C – Compound **2**



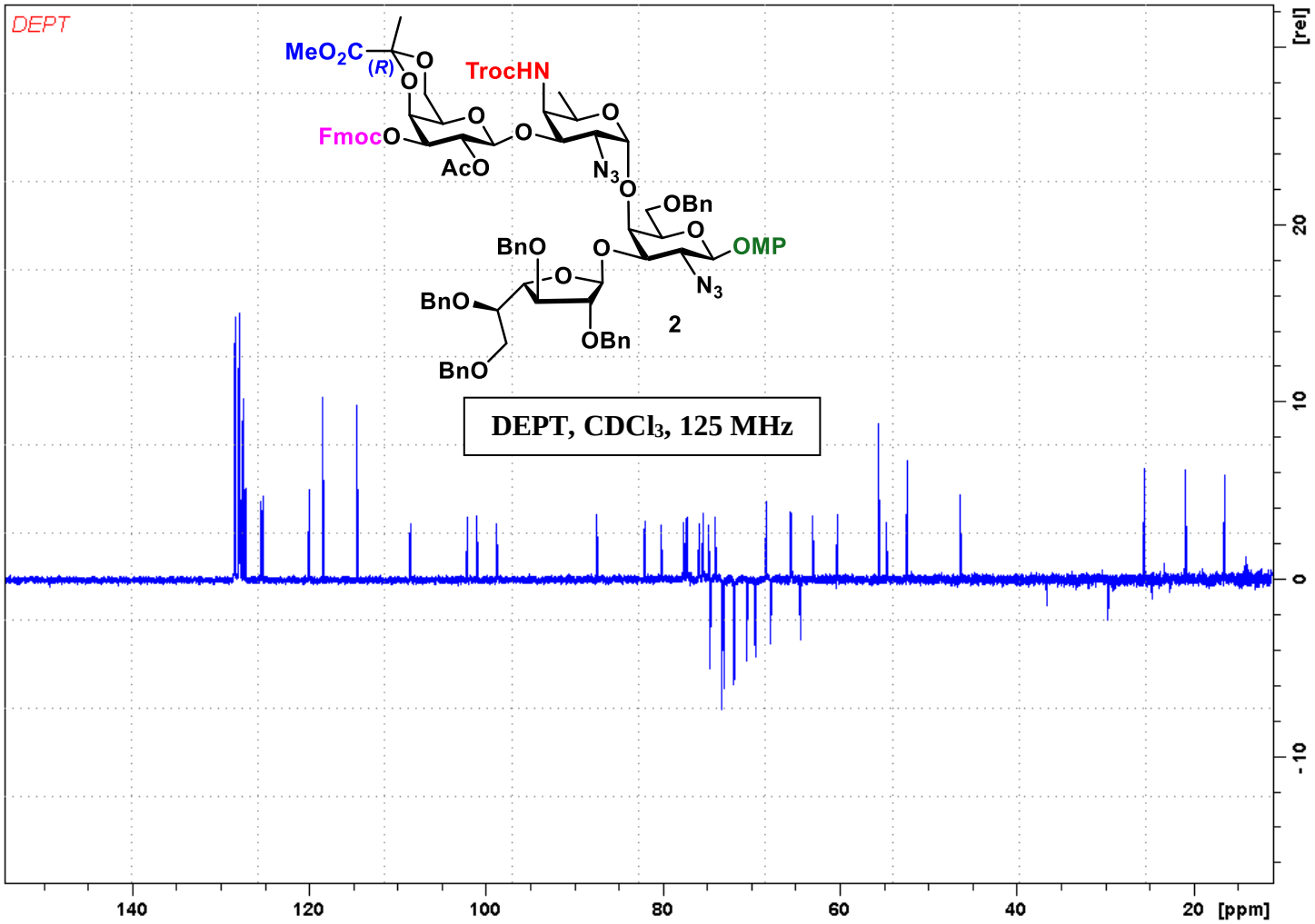
COSY – Compound **2**



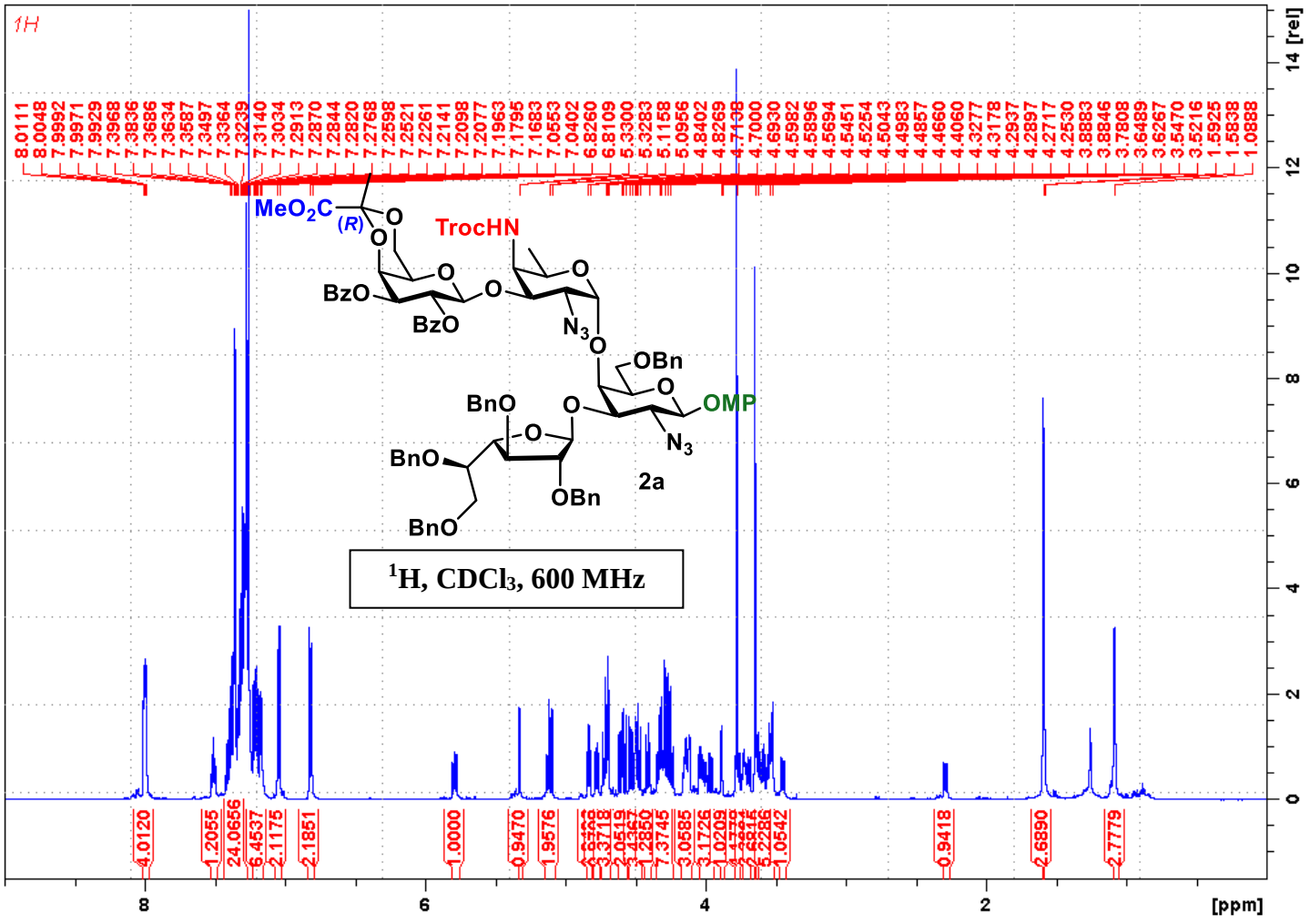
HSQC – Compound **2**



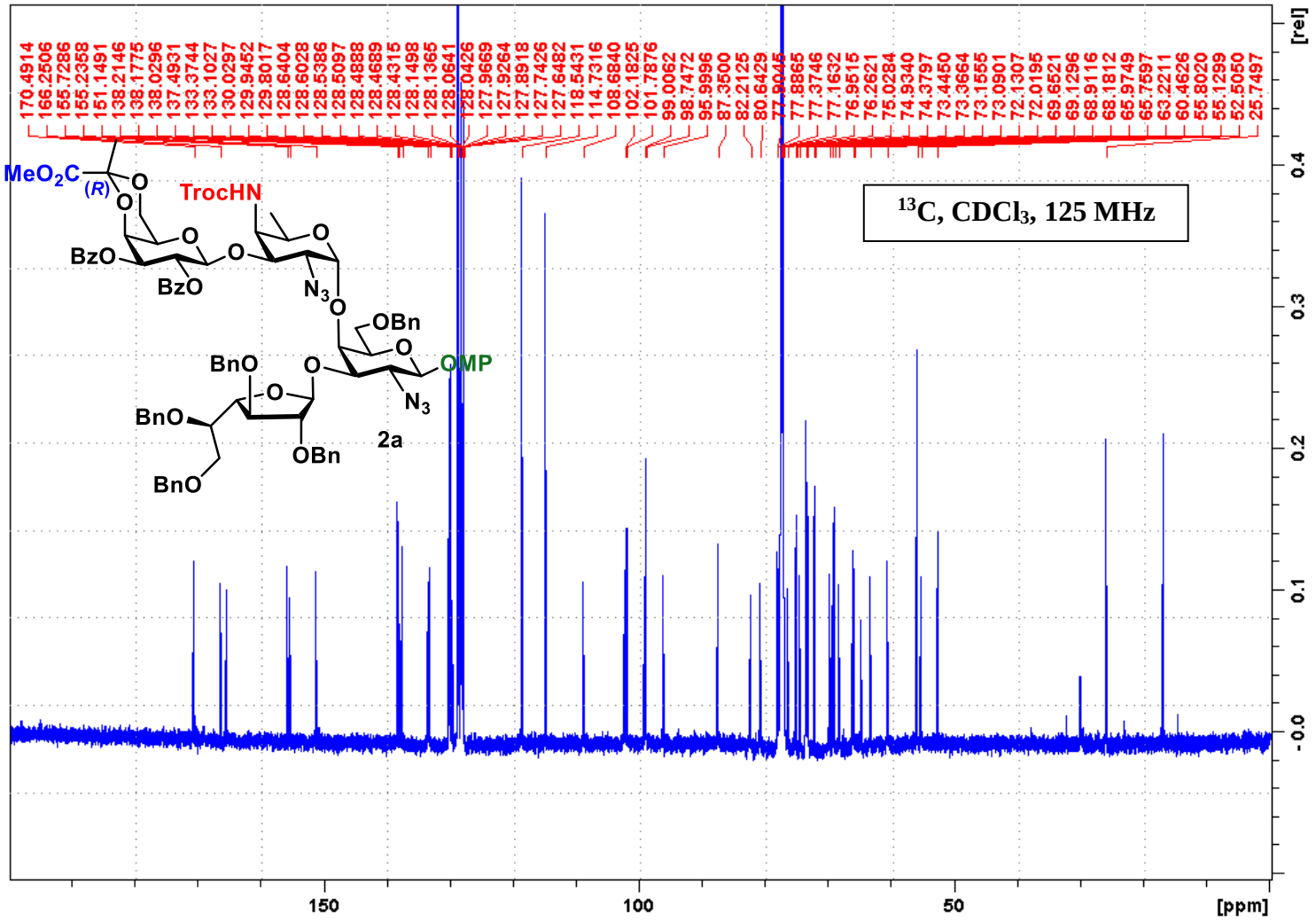
DEPT – Compound **2**



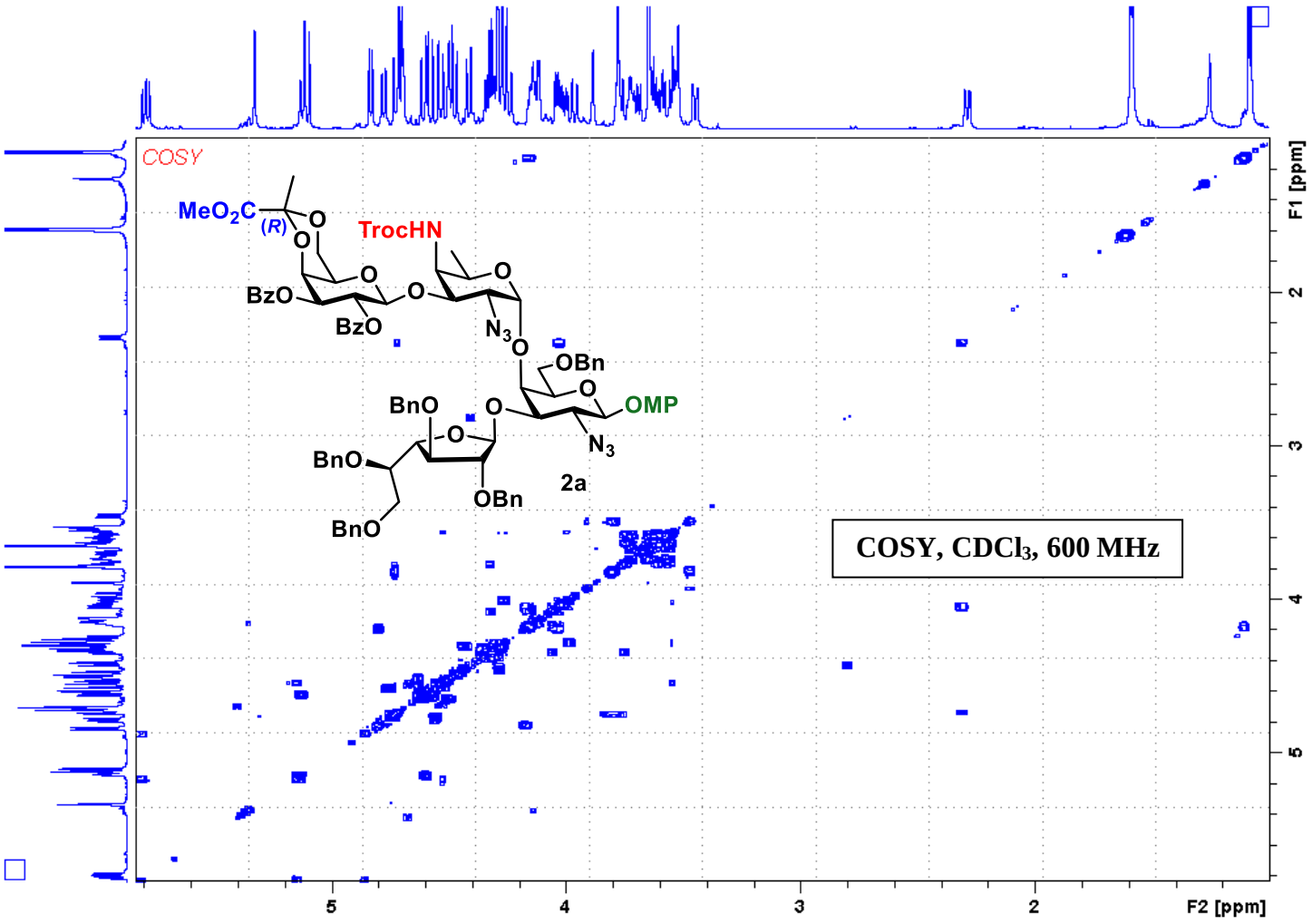
$^{1}$H – Compound **2a**



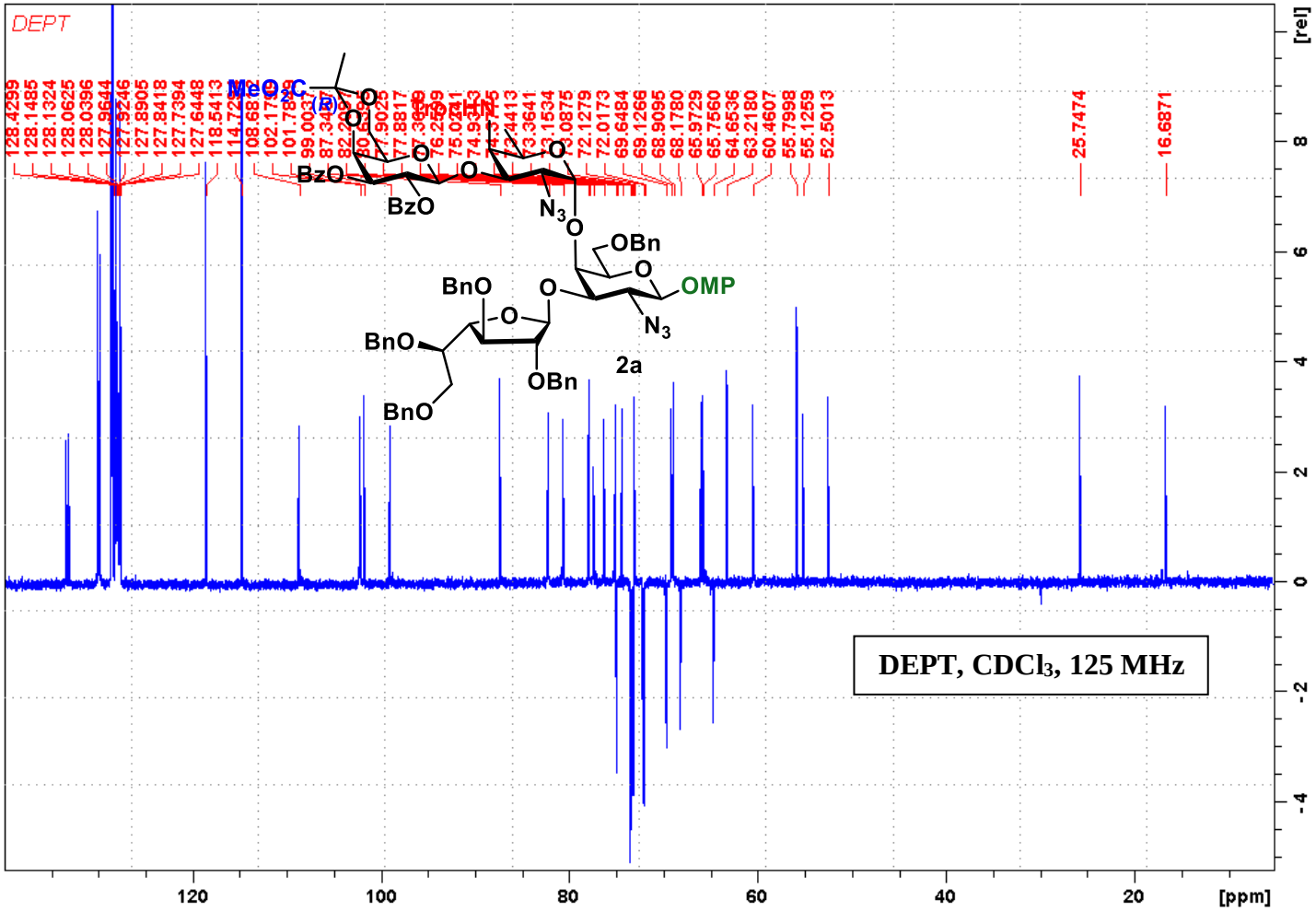
$^{13}$C – Compound **2a**



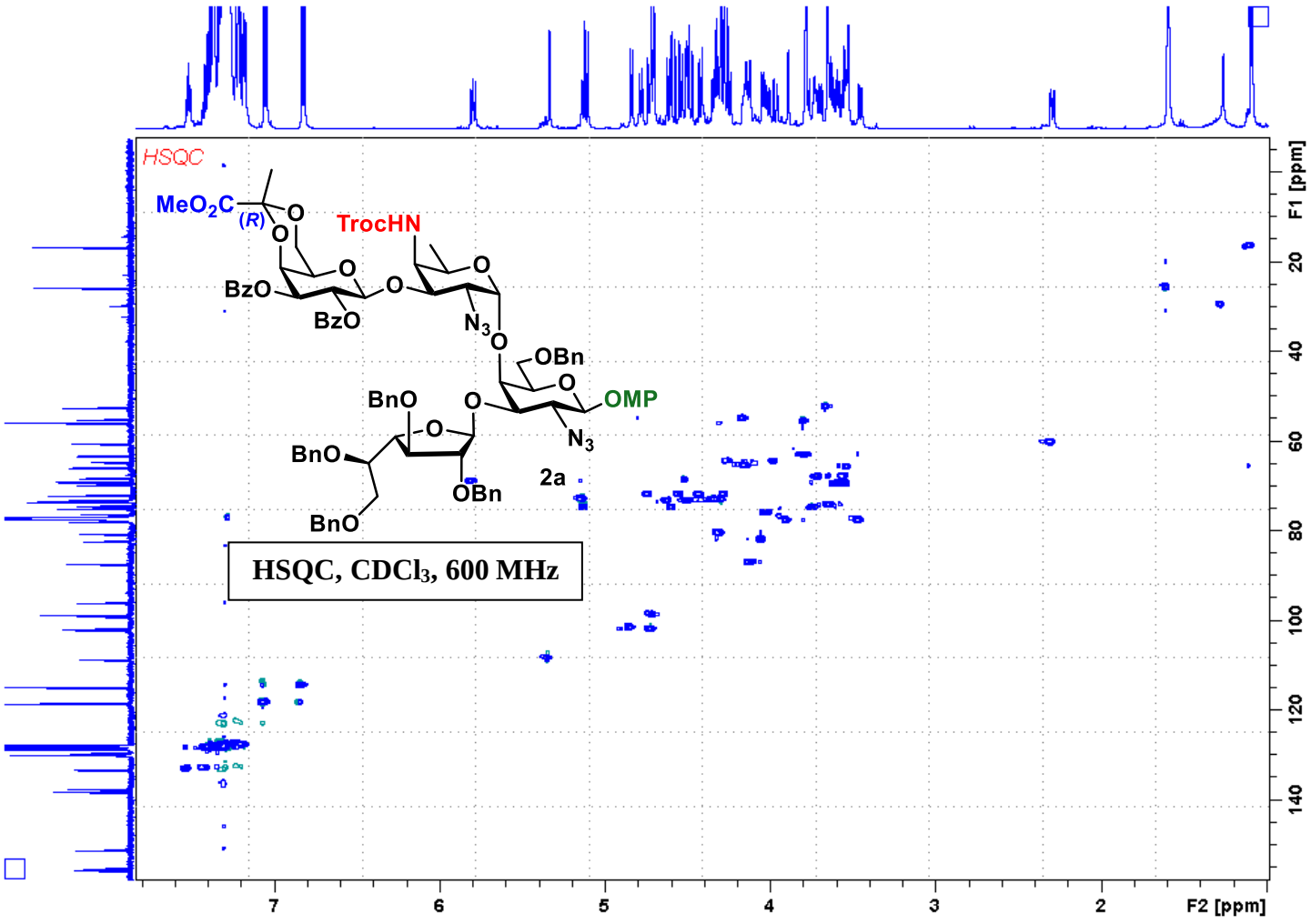
COSY – Compound **2a**



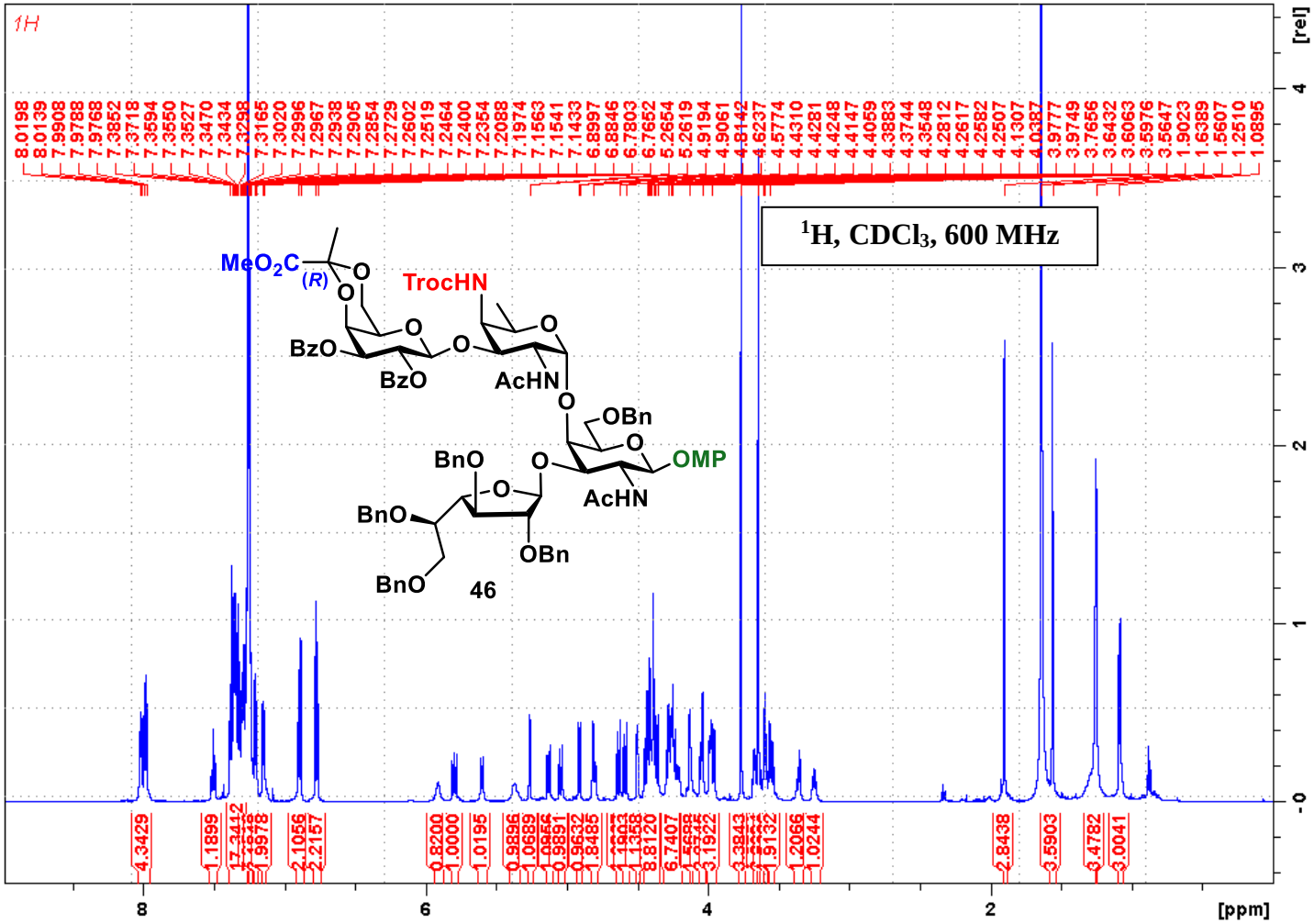
DEPT – Compound **2a**



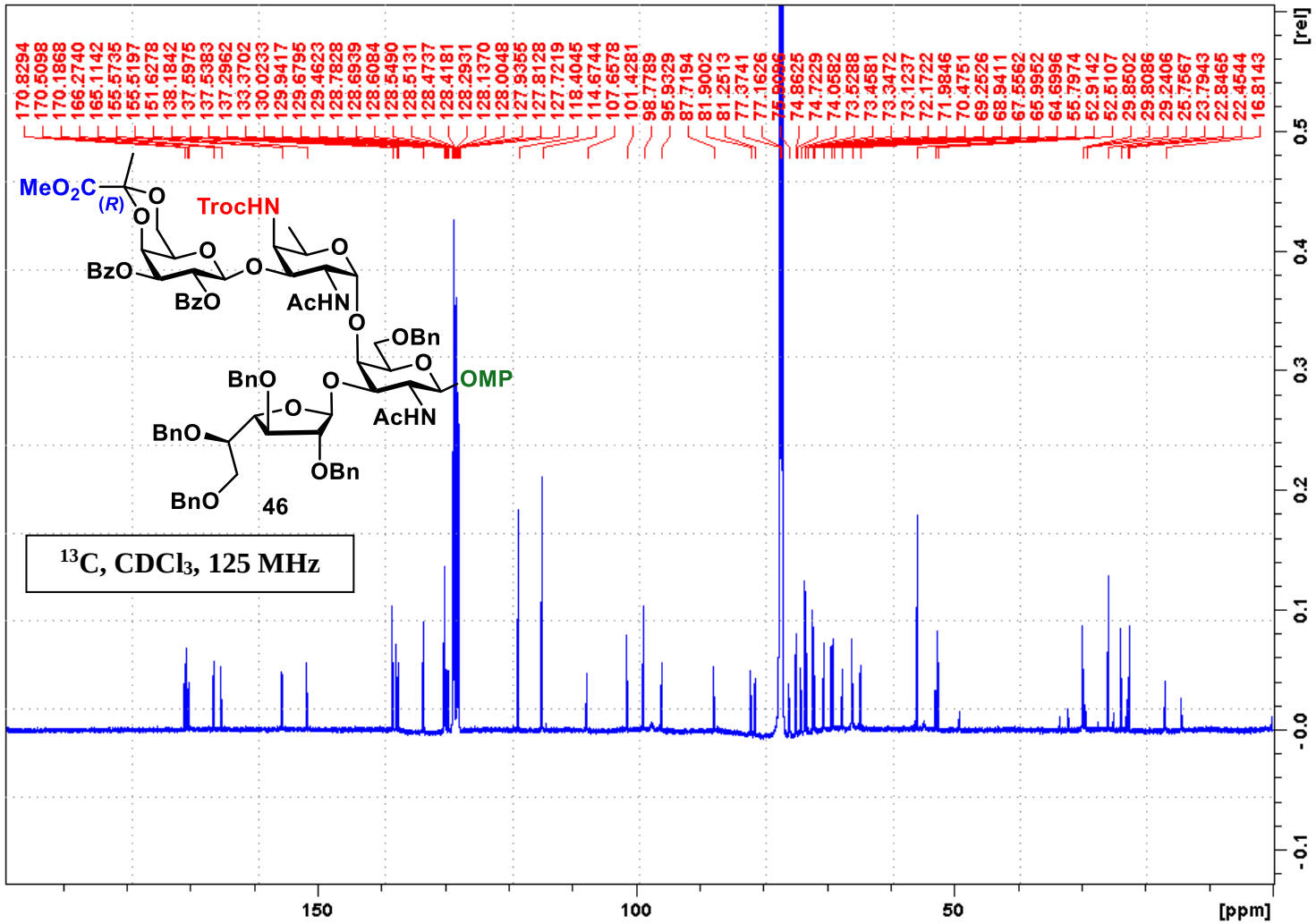
HSQC – Compound **2a**



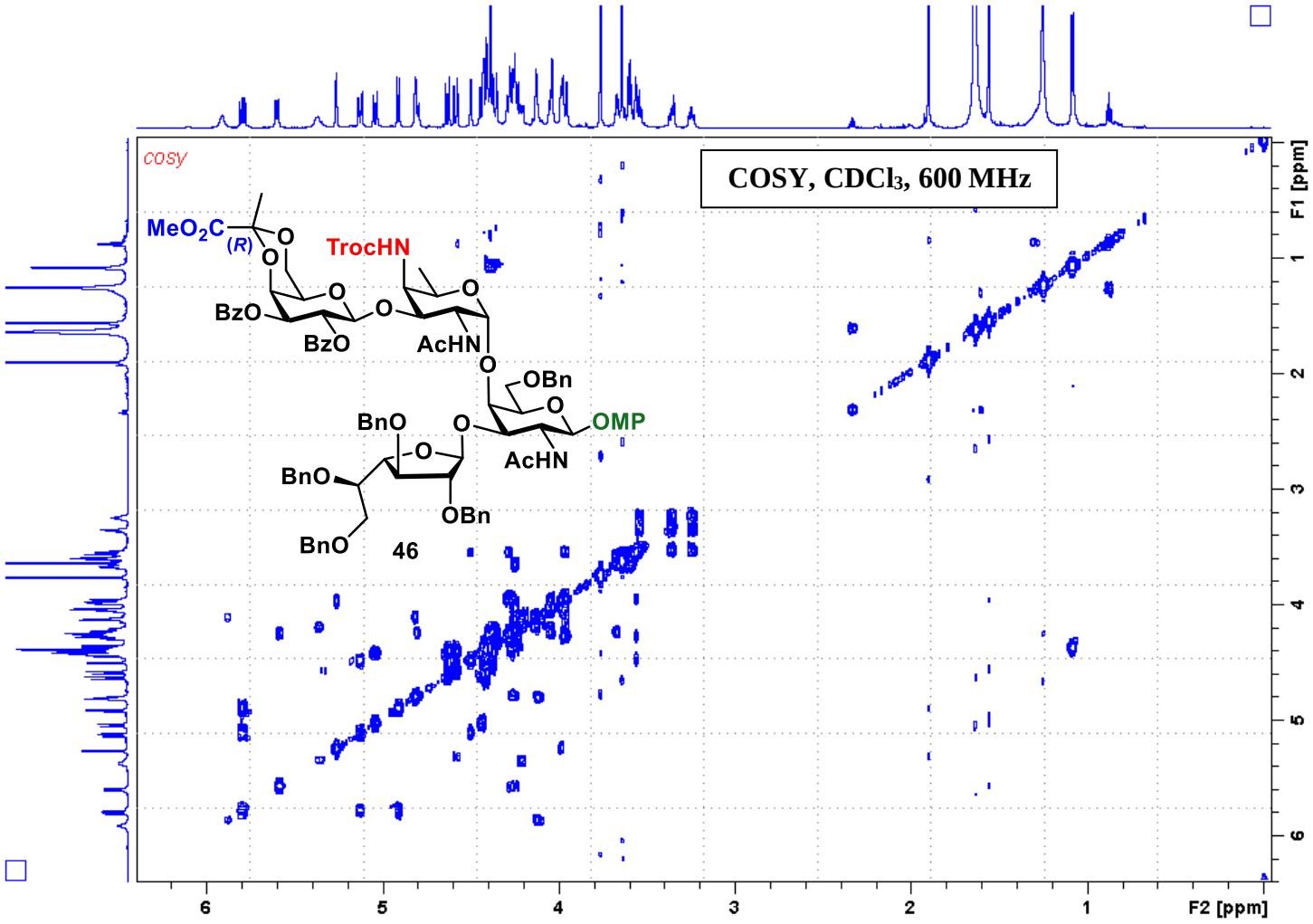
$^{1}$H – Compound **46**



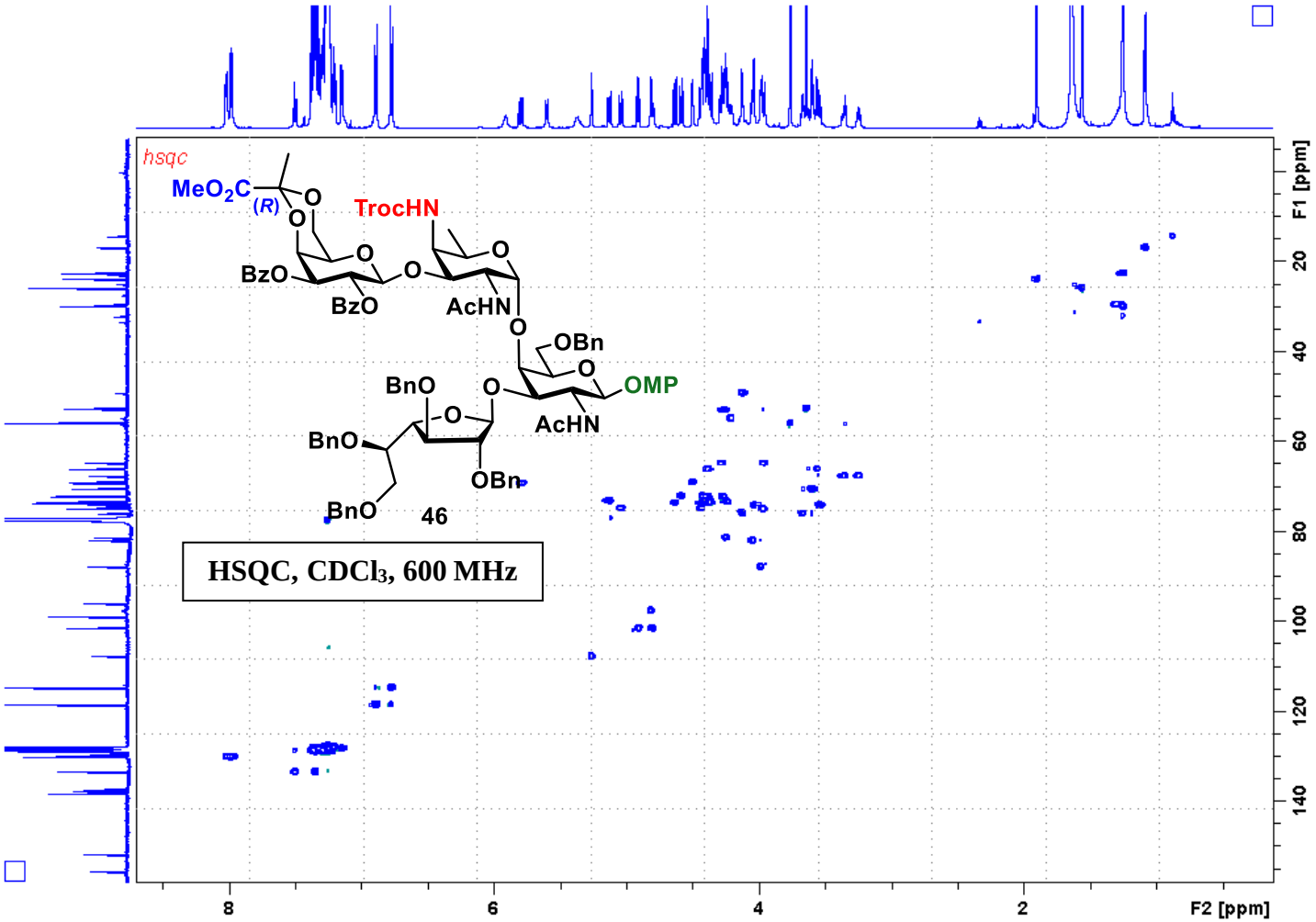
$^{13}$C – Compound **46**



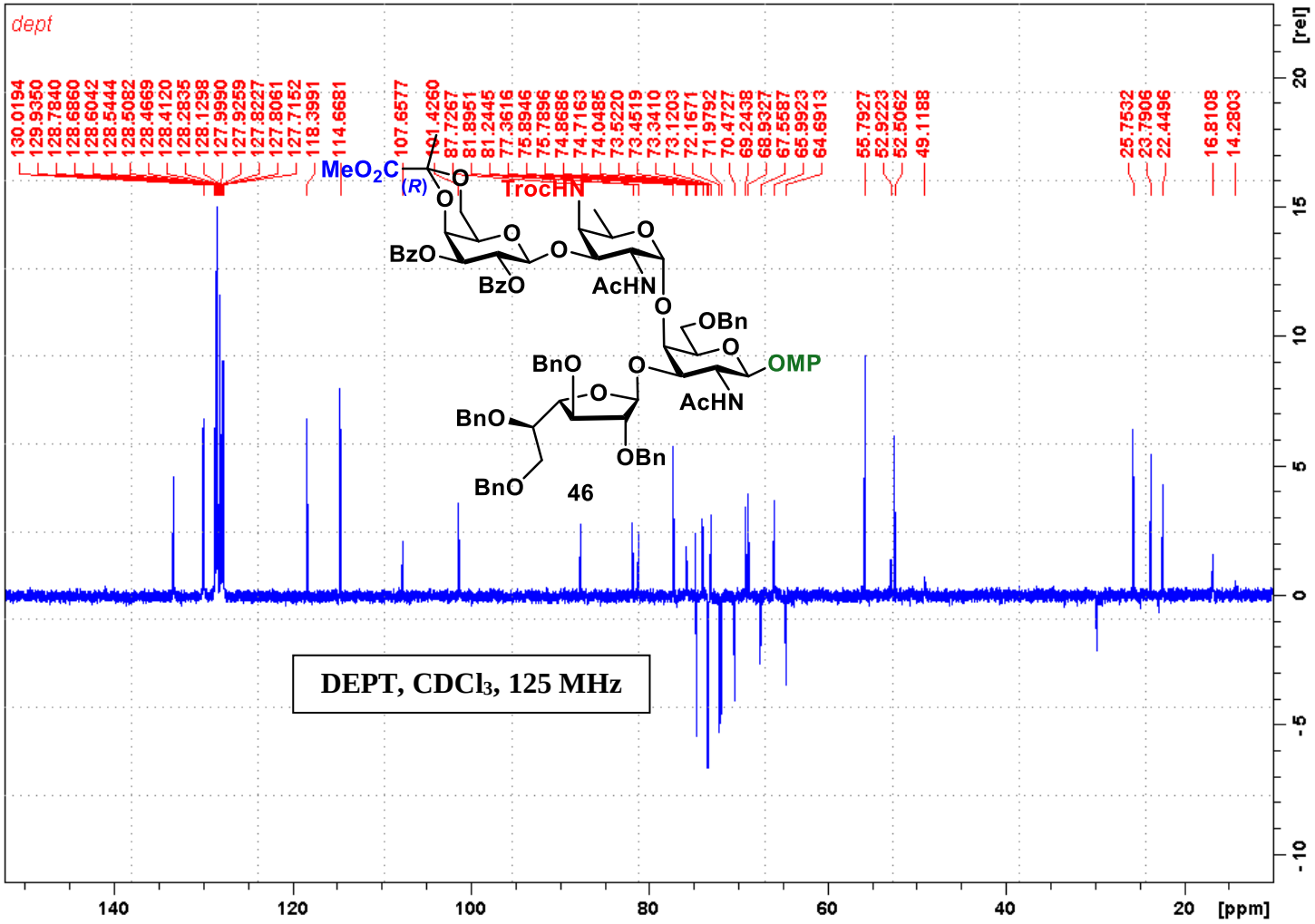
COSY – Compound **46**



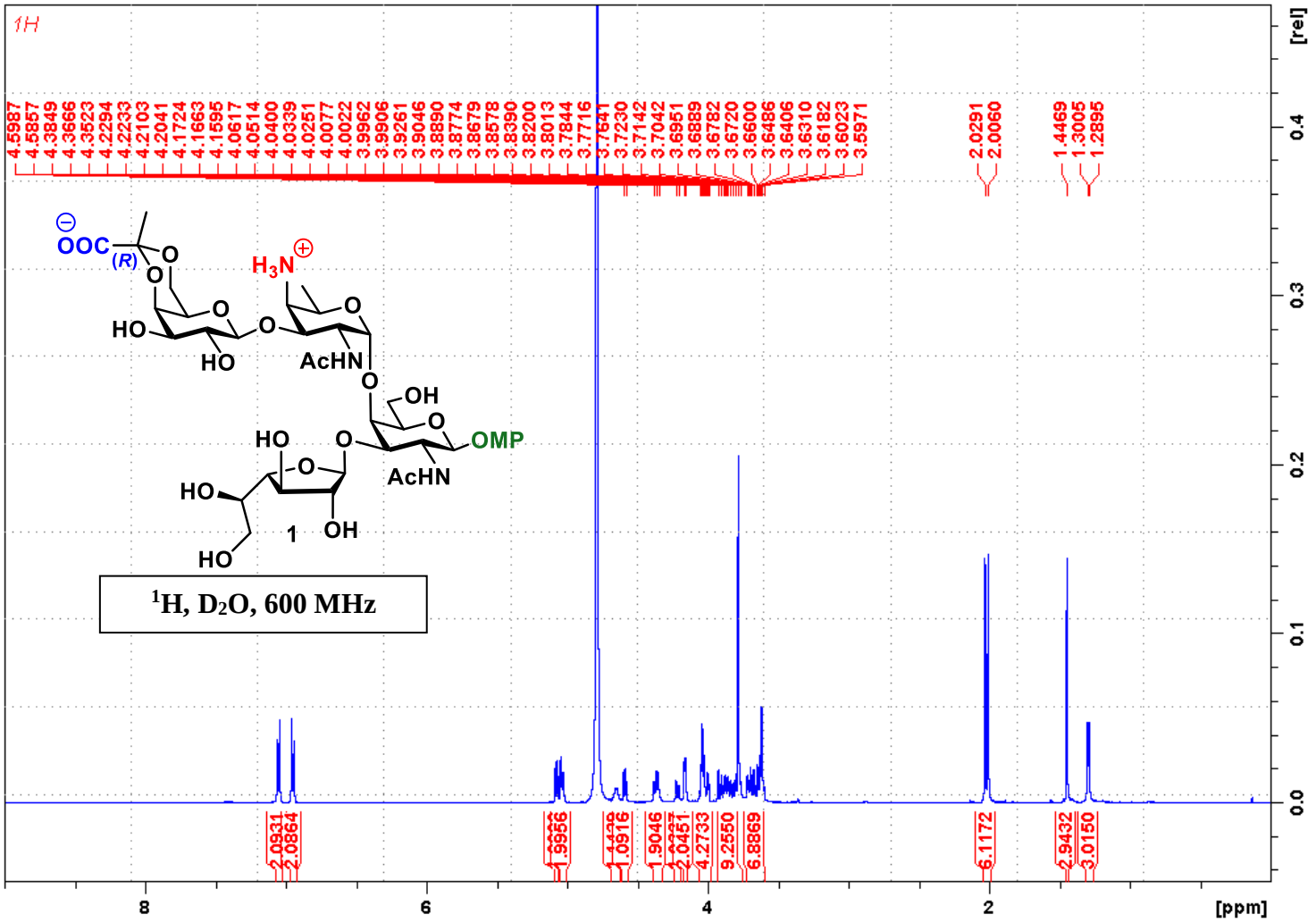
HSQC – Compound **46**



DEPT – Compound **46**

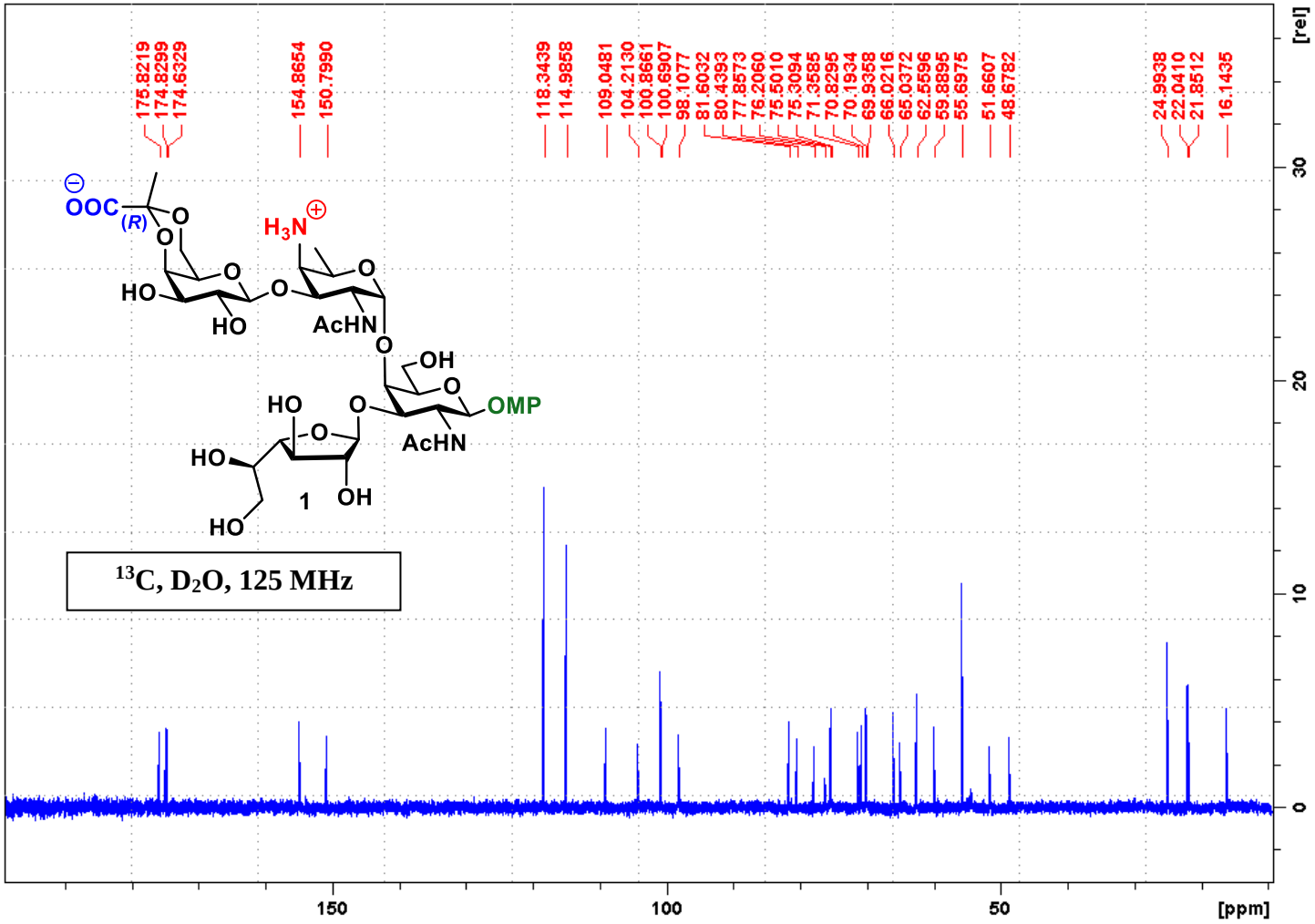


$^{1}$H – Compound **1**




**$^{1}$H, $D_2O$, 600 MHz**

$^{13}$C - Compound **1**



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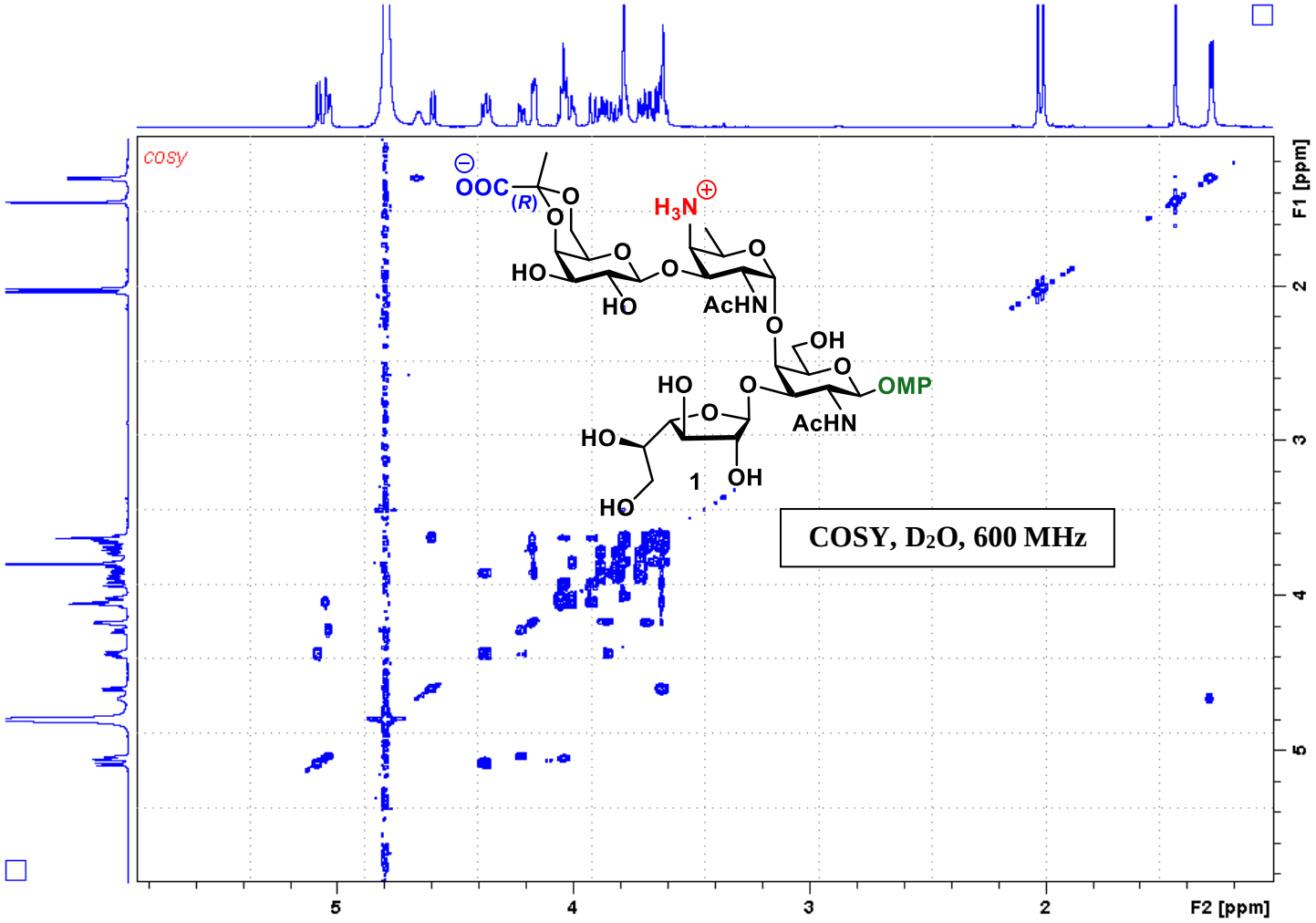

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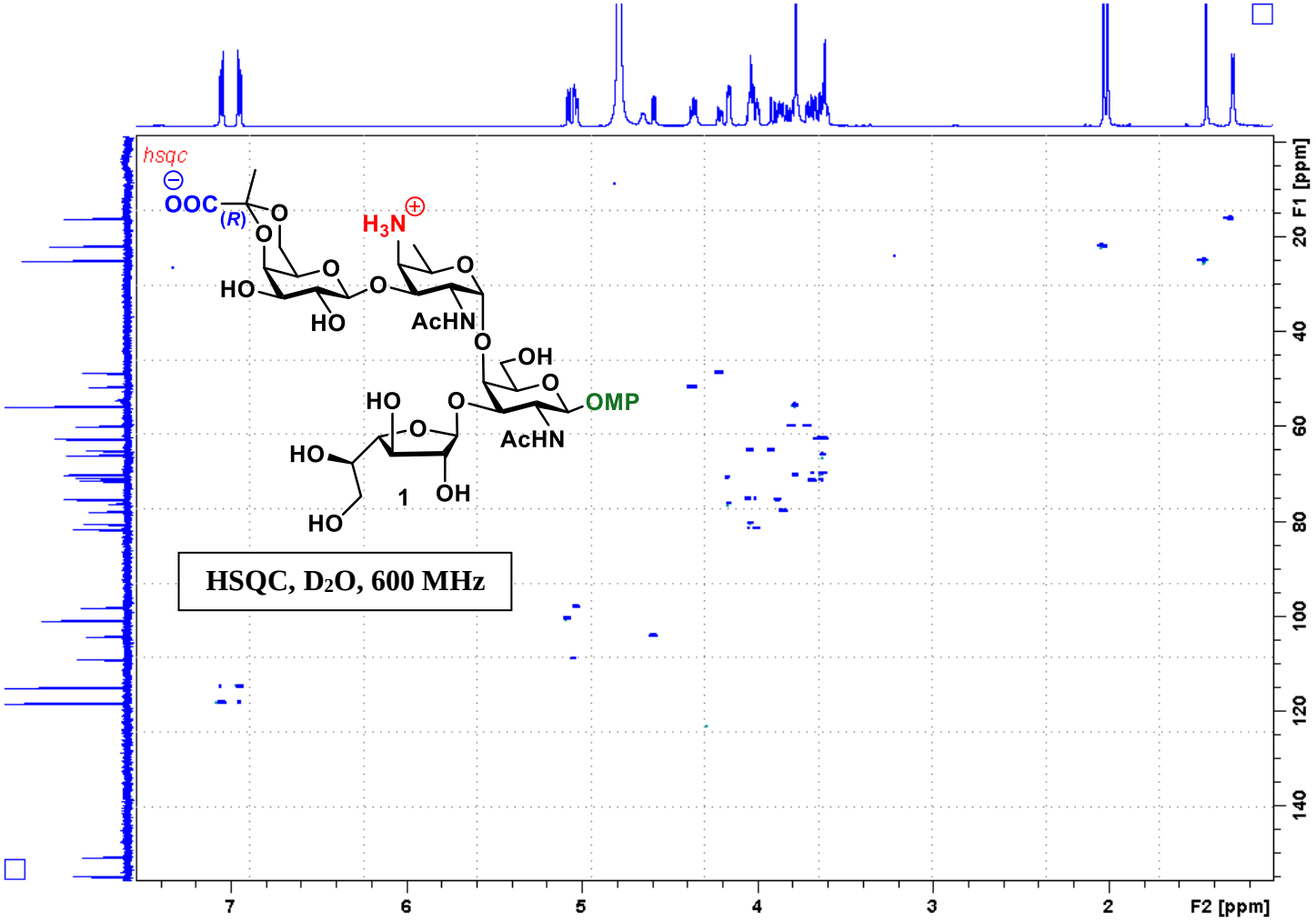

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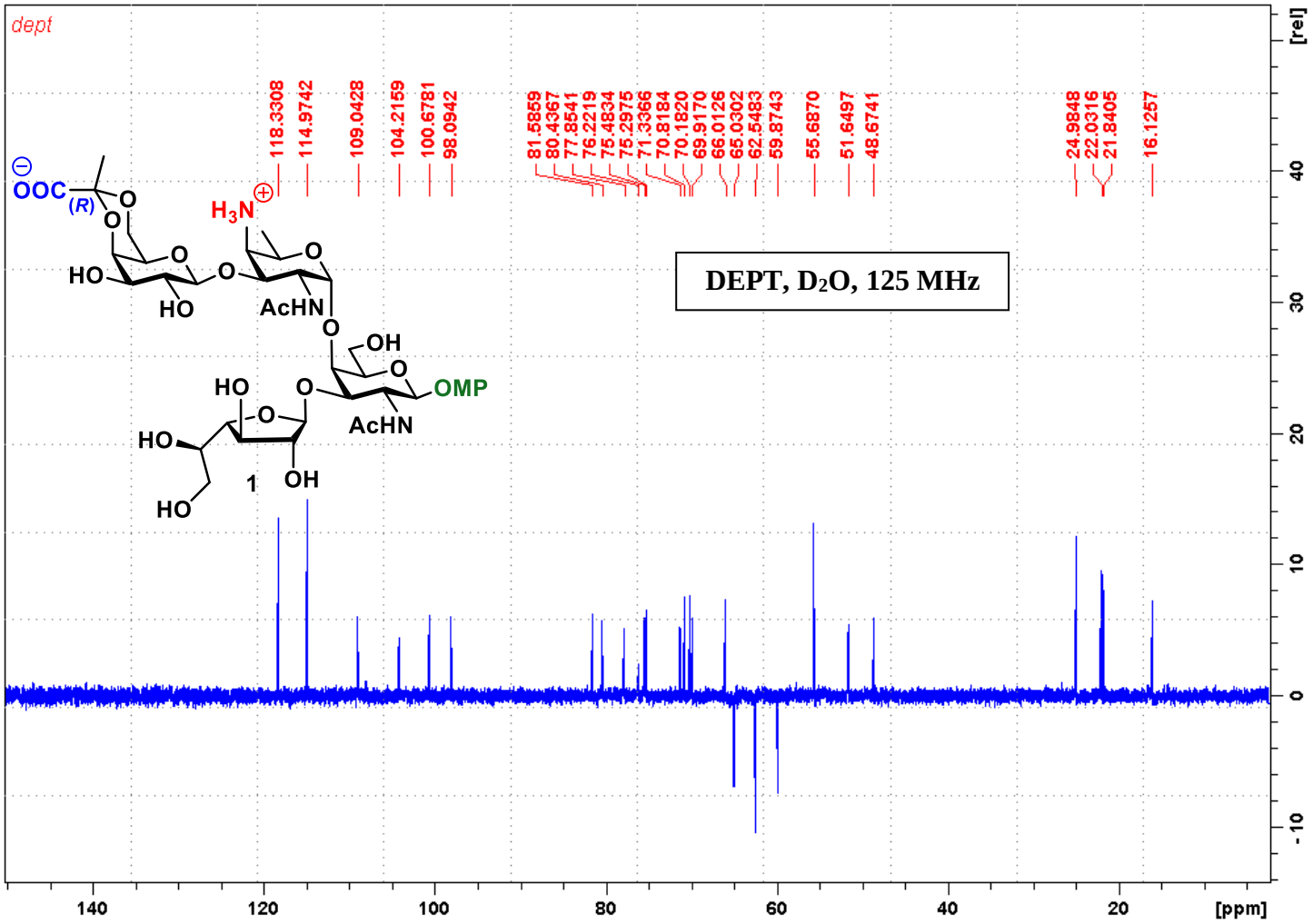
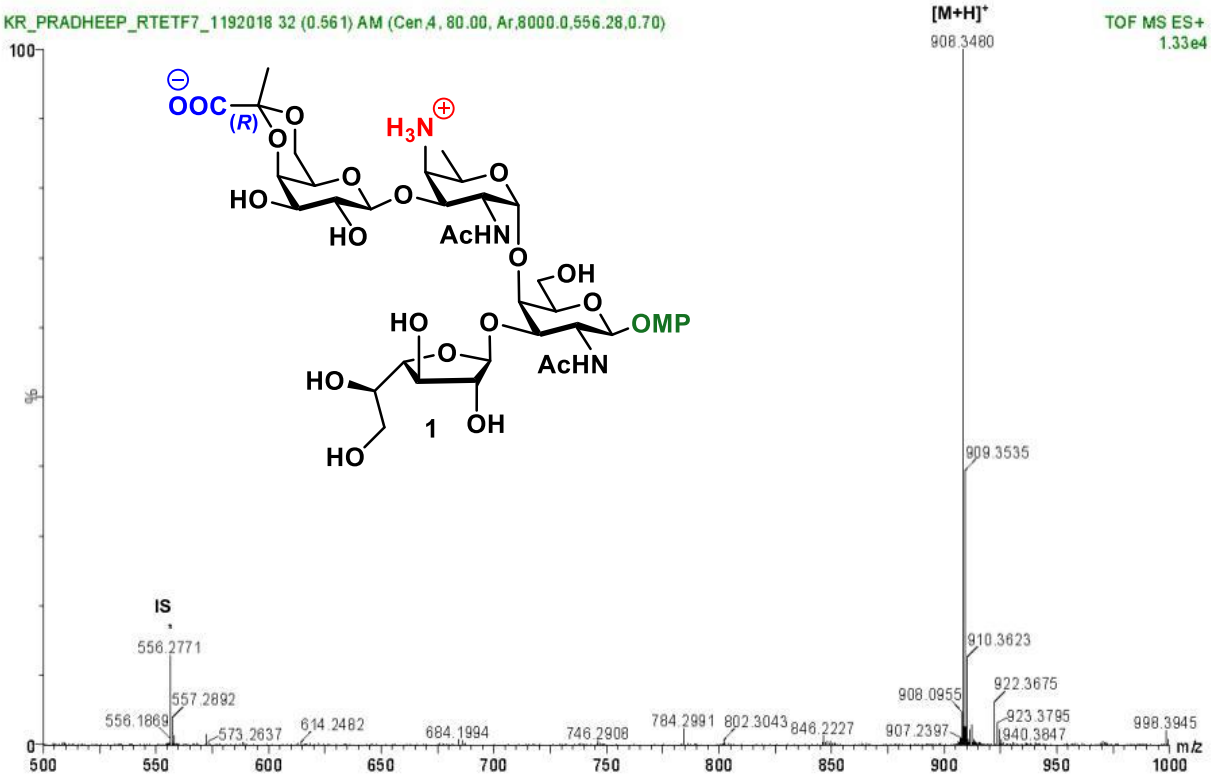


R-TET-F7




Mass accuracy = ((908.3512-908.3480)/908.3512)* $10^6$ = 3.5 ppm

# Chapter 3

# Synthesis of Zwitterionic Polysaccharide A1 (PS A1) Antigenic Units of Oligomeric length

## 3.1. Introduction:

In order to understand the T-cell activation mechanism by the zwitterionic polysaccharides (ZPSs), homogenous units or fragments of PS A1 are required. Biological isolation of PS A1 from the culture of *B. fragilis* NCTC 9343 results in heterogeneous polysaccharide fragments, not suitable as molecular probes to investigate the critical details of the T-cell activation pathway. Synthesis of PS A1 repeating units can solve the issue of heterogeneity and can provide structurally defined homogenous material in sufficient quantities. The first step in regard to the identification of the immunologically active epitope of polysaccharide A1 (PS A1) with an ability to activate $CD4^+$ T-cell is to obtain access to pure synthetic fragments with three possible *sequential* epitopes and higher molecular weight (>3 kDa) *conformational* epitope antigen fragments.

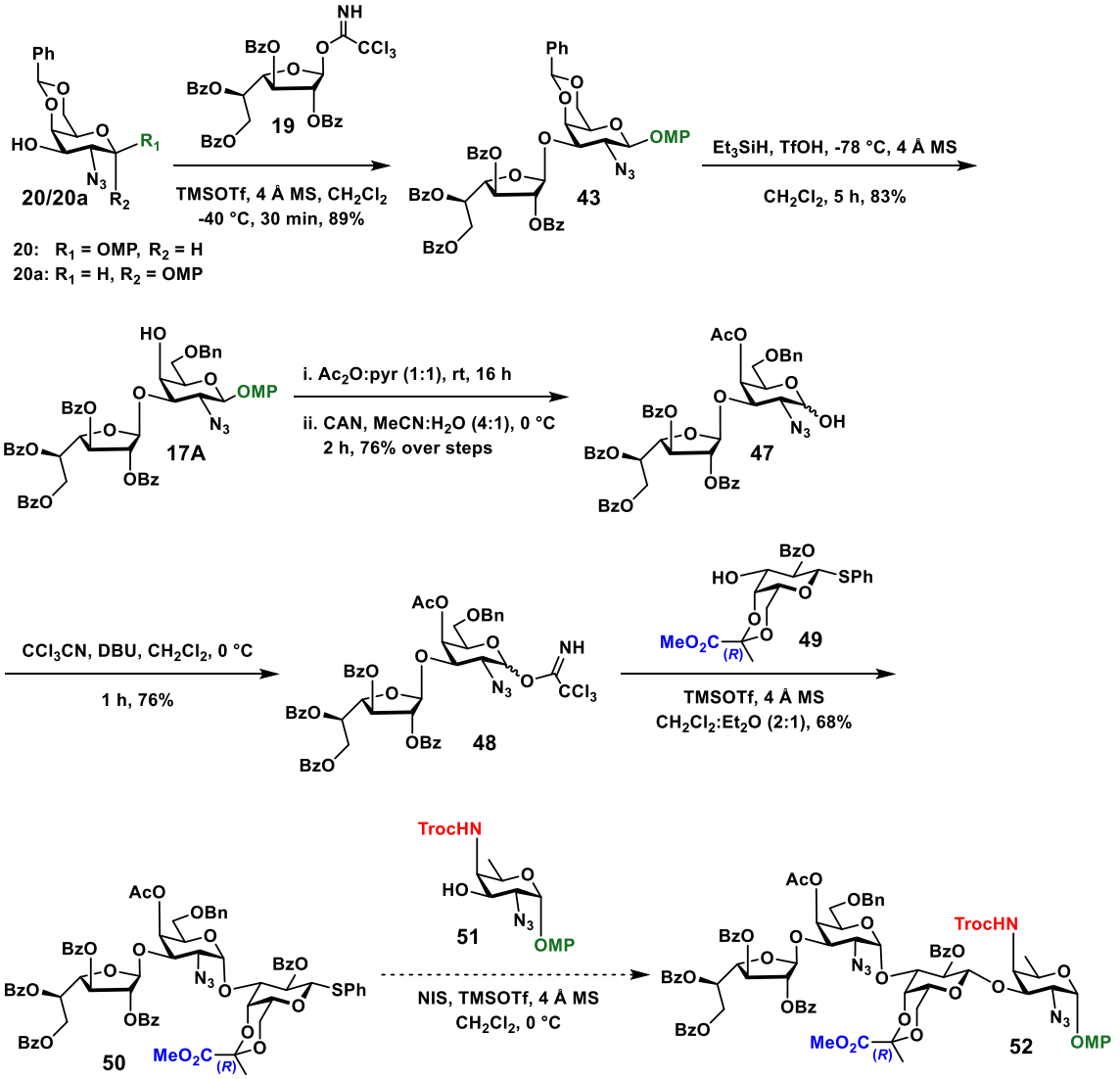
With the above mentioned synthetic antigenic fragments of PS A1 in hand, *in vitro* studies like MHC II binding with HLA-DR2 using ITC, T-cell proliferation assay and *in vivo* studies in mice can be executed using both *sequential* and *conformational* synthetic

PS A1 antigen fragments. From both *in vitro* and *in vivo* studies, the effect of sequence and conformation over the process of activation of T-cell can be understood in a clear-cut fashion, providing information regarding a very important structural feature required for the T-cell activation apart from the zwitterionic property of PS A1 antigens. Hence, with various synthetic building blocks in hand, attempts towards the synthesis of *sequential* and *conformational* epitopes of polysaccharide A1 (PS A1) was initiated. Except the rare D-AAT sugar, other three building blocks were synthesized in large scale to benefit the future access to oligomeric length PS A1 fragments.

### 3.2. Synthesis of zwitterionic repeating unit with frameshift C:

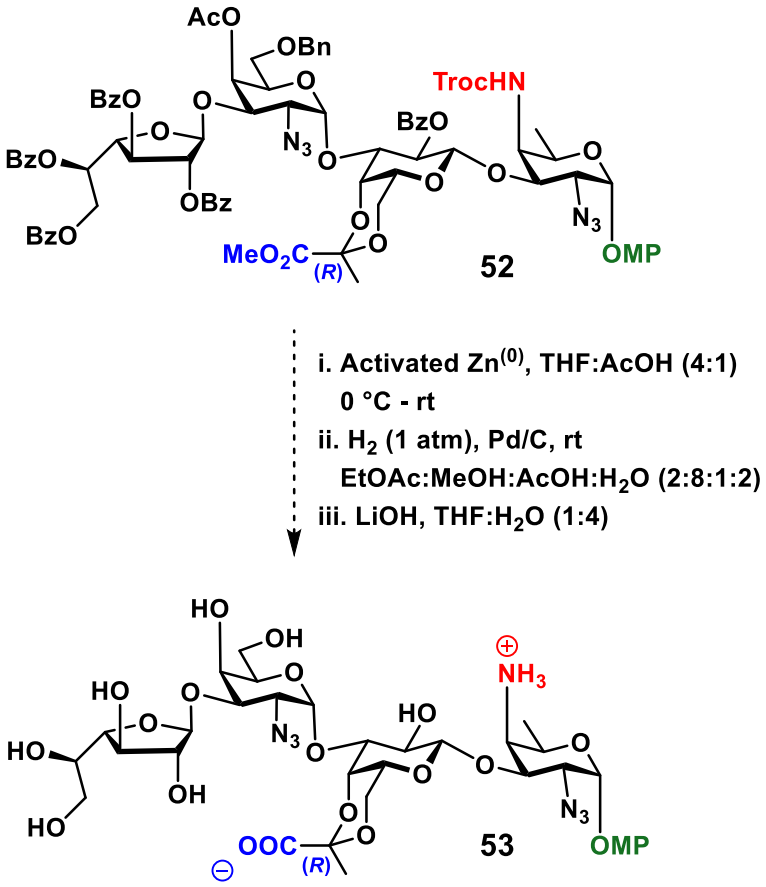
In order to access the *sequential* and *conformational* epitopes, monosaccharide building blocks were protected using various protecting groups to afford the respective donor and acceptors. To access the frameshift C, linear glycosylation approach was used adapting the previous protocol (frameshift B). Synthesis of the zwitterionic repeating unit **53** with frameshift C started with [1+1] glycosylation using the D-Gal*f* trichloroacetimidate donor **19** and 4,6-*O*-benzylidene protected D-$GalN_3$ acceptor **20** (Scheme 10). The TCA donor **19** and D-$GalN_3$ acceptor **20** were coupled using TMSOTf as activator in dichloromethane at -40 ºC to afford the disaccharide **43** in 89% yield. The disaccharide **43** was then subjected to regioselective benzylidene ring opening using TfOH and triethylsilane ($Et_3SiH$) in dichloromethane at -78 ºC to afford the free C4-OH disaccharide **17A** in 83% yield (Scheme 10). The respective C4-OH disaccharide **17A** was protected with acetyl protecting group using acetic anhydride ($Ac_2O$)/pyridine, followed by the *p*-methoxy phenol group deprotection at the reducing end to afford the disaccharide hemiacetal **47** in 76% yield over two steps. The hemiacetal **47** was then converted to TCA

donor using $CCl_3CN$ and cat. DBU in dichloromethane to afford the desired TCA donor **48** in 76% yield.




**Scheme 10:** Synthesis of protected PS A1 tetrasaccharide **52** with frameshift C.

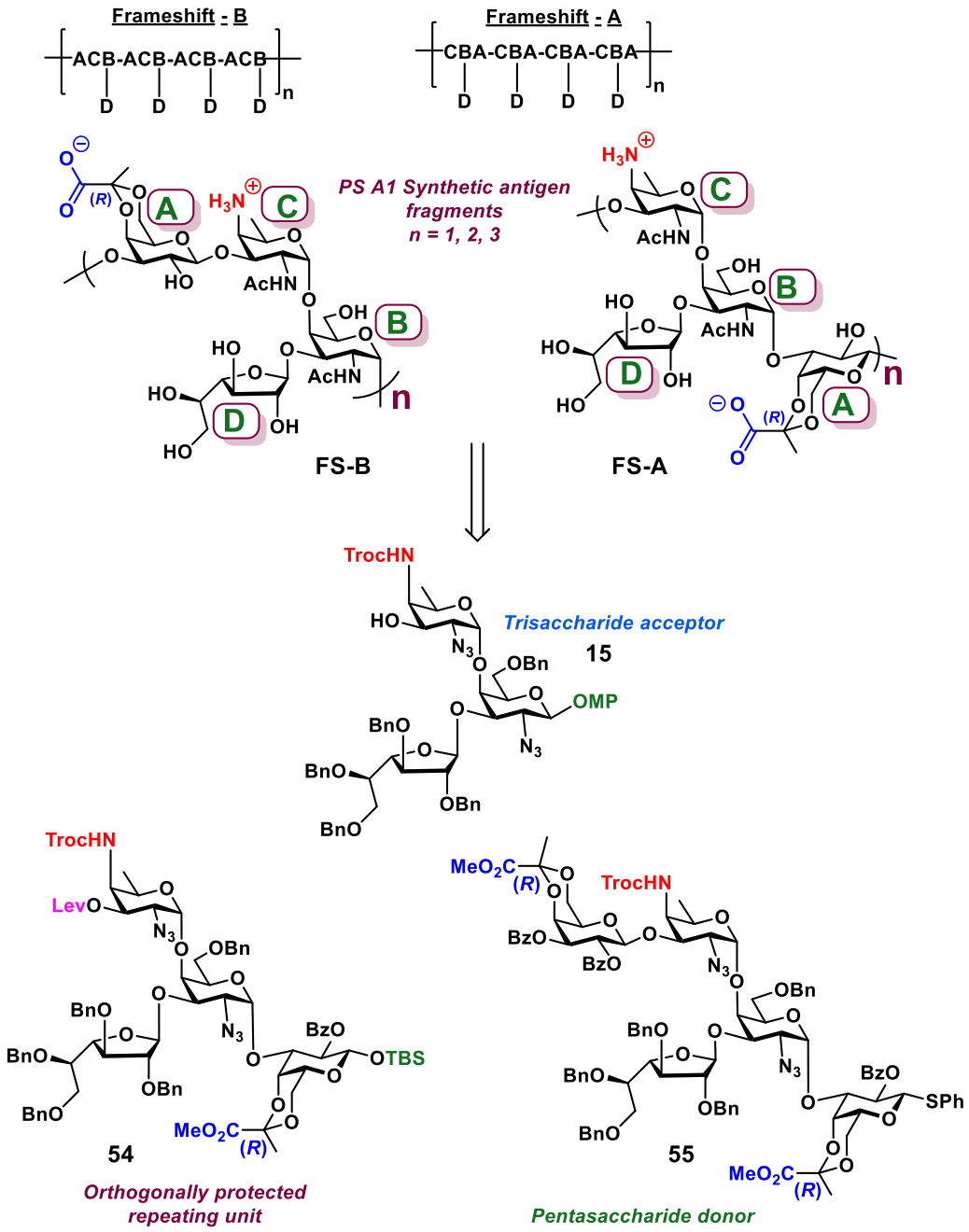
The disaccharide TCA donor **48** was coupled with C3-OH pyruvate D-Gal*p* acceptor **49**, using *N*-iodo succinimide (NIS) and TMSOTf in a 2:1 mixture of dichloromethane and diethyl ether ($Et_2O$) to afford the trisaccharide **50** in 68% yield.




**Scheme 11:** Synthesis of zwitterionic tetrasaccharide **53** with frameshift C.

The trisaccharide thiophenyl donor **50** can be used to couple with α-*p*-methoxy phenyl C3-OH D-AAT acceptor **51** to afford the desired protected tetrasaccharide repeating unit of polysaccharide A1 (PS A1) **52** with frameshift C using the C2-OBz anchimeric assistance in the donor (Scheme 10). The global deprotection (Scheme 11) of the respective protected tetrasaccharide **52** using Zn/AcOH in THF to deprotect the Troc protecting group, followed by the Pd/C hydrogenation to remove benzyl protecting group and final LiOH mediated saponification reaction to deprotect the esters will afford the desired completely deprotected zwitterionic tetrasaccharide **53** with frameshift C.

### 3.3. Synthesis of oligomeric length PS A1 zwitterionic fragments




**Scheme 12:** Retrosynthetic analysis to access oligomeric PS A1 repeating units

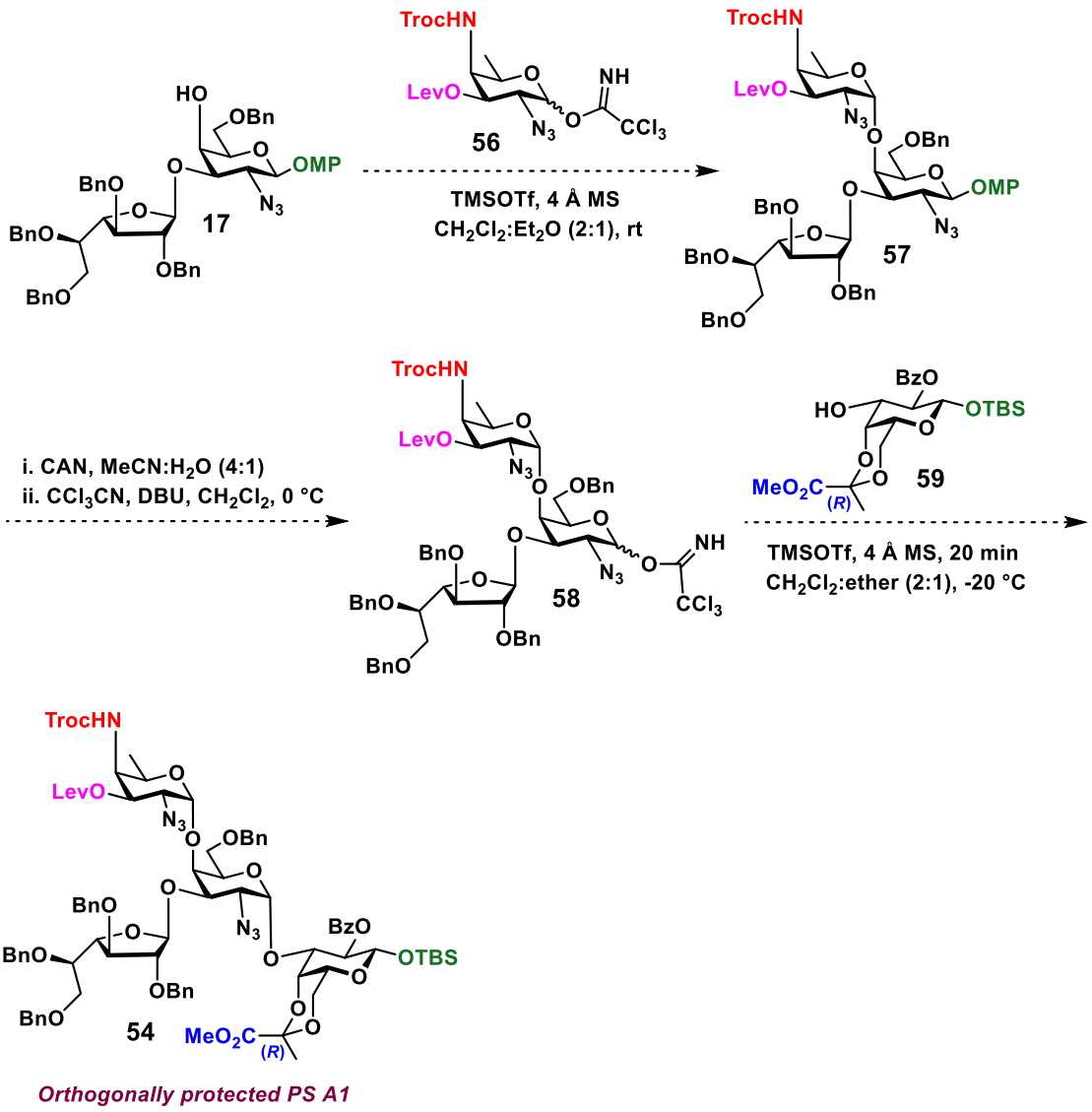
Synthesis of oligomeric length fragments can be accessed from three PS A1 synthetic fragments trisaccharide acceptor **15**, orthogonally protected tetrasaccharide **54** and pentasaccharide fragment **55** (Scheme 12). For example, the trisaccharide acceptor **15**

and pentasaccharide donor **55** can be coupled to obtain the octasaccharide (two repeating unit fragment). Similarly, the coupling of trisaccharide acceptor **15** and TCA donor of orthogonally protected tetrasaccharide **54** can provide a heptasaccharide, followed by the [5+7] glycosylation affords the three repeating unit sequence of PS A1 with ACB-ACB frameshift (Scheme 12). The orthogonally protected tetrasaccharide repeating unit **54** by itself can provide access to both tetrasaccharide acceptor and donor based on the chemoselective deprotection of the Lev and TBS protecting groups. The respective tetrasaccharide donor and acceptor can be used to gain access to octasaccharide with a different frameshift. The importance of sequence or frameshift might diminish as the number of repeating units in the antigenic fragment of PS A1 increase (PS A1 oligomers > 3kDa), as the oligomeric PS A1 fragments can adopt helical conformation placing the zwitterionic positive and negative charges on opposite sides of the helical conformation.

### 3.3.1. Synthesis of orthogonally protected tetrasaccharide 54:

The trisaccharide fragment **15** can be accessed using the previous protocol (Scheme 7), orthogonally protected tetrasaccharide repeating unit **54** can be synthesized starting with [2+1] glycosylation using disaccharide acceptor **17** and C3-lev protected D-AAT donor **56**. The trisaccharide **57** can then be subjected to OMP group deprotection, followed by treatment with $CCl_3CN$ and cat. DBU to afford the trisaccharide TCA donor **58**. Using a [3+1] glycosylation strategy, the TCA donor **58** and C3-OH pyruvate acceptor **59** can be coupled to obtain the orthogonally protected PS A1 repeating unit **54** (Scheme 13). The orthogonally protected tetrasaccharide repeating unit **54** with TBS and Lev protecting groups at the reducing and non-reducing ends can be chemo-selectively deprotected to obtain tetrasaccharide acceptor, donor and can be coupled using [4+4] glycosylation to gain

access to orthogonally protected octasaccharide repeating unit and so on. The final global deprotection will lead to the formation of completely deprotected oligomeric length fragments of PS A1.

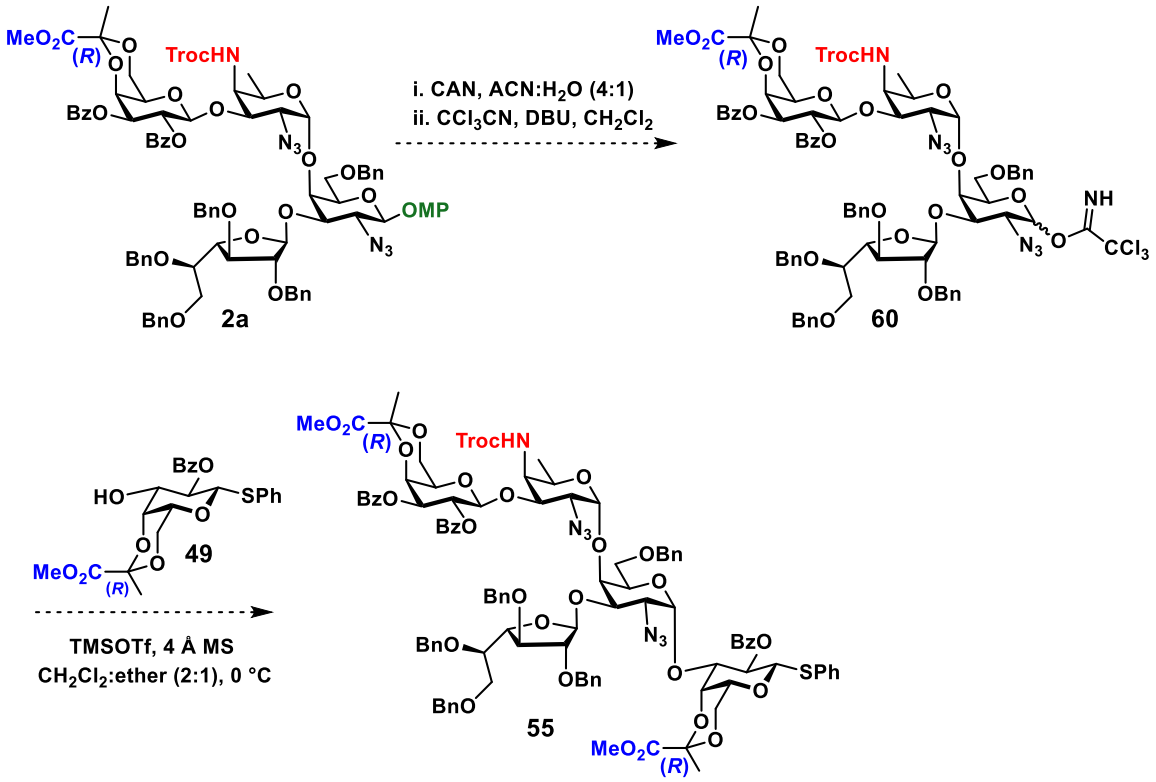



**Scheme 13:** Synthesis of orthogonally protected tetrasaccharide repeating unit **54**

### 3.3.2. Synthesis of pentasaccharide donor 55:

The OMP protected tetrasaccharide **2a** at the reducing end can be converted to TCA donor **60** in two steps, followed by [4+1] glycosylation with C3-OH pyruvate D-Gal*p*

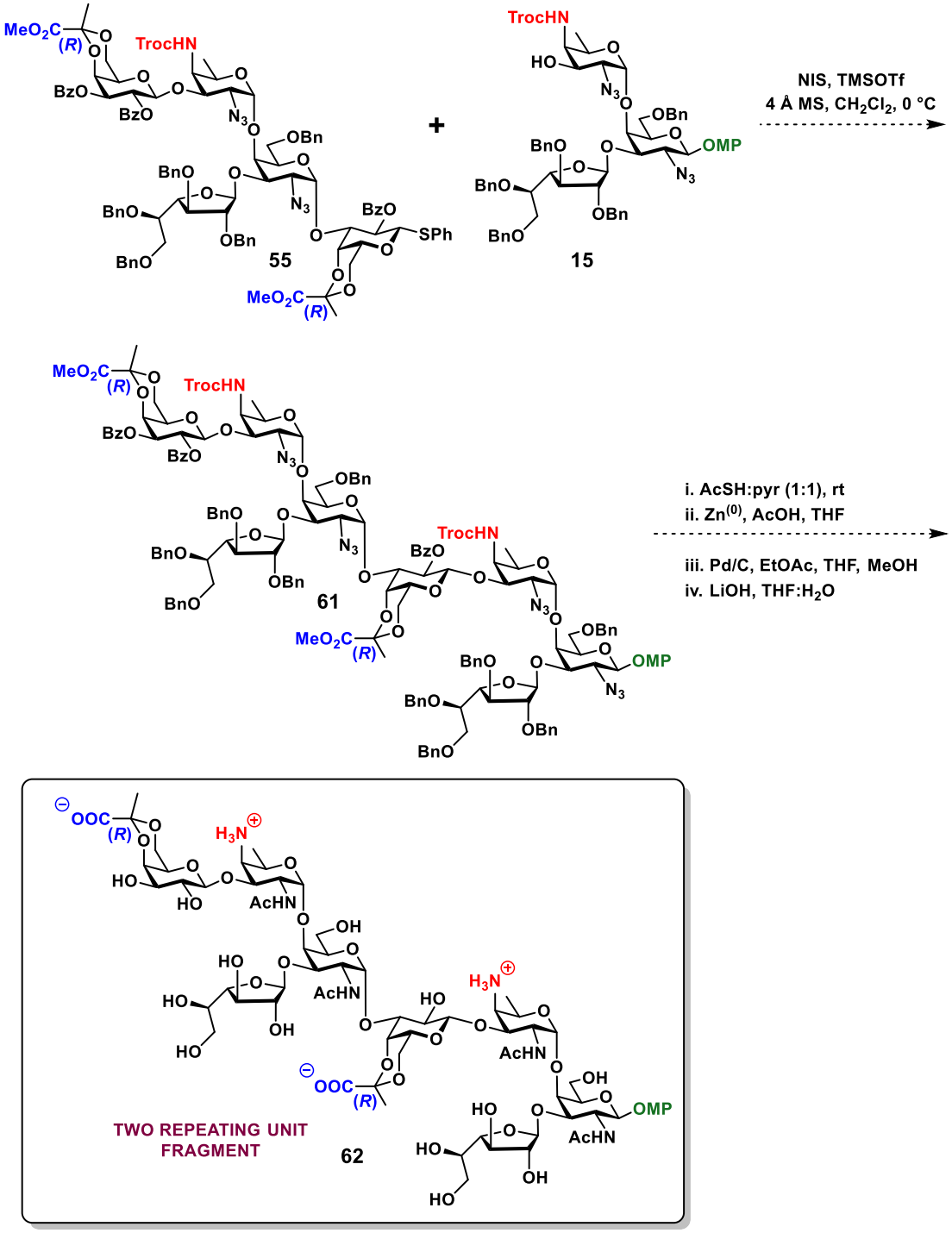
acceptor **49** to afford the pentasaccharide **55** (Scheme 14). The above three synthetic fragments of PS A1 can now be used to access oligomeric length PS A1 repeating units with two different frameshifts (A and B).




**Scheme 14:** Synthesis of pentasaccharide donor **55** of PS A1

### 3.3.3. Synthesis of octasaccharide fragment 62 of PS A1:

The two repeating unit or octasaccharide fragment of PS A1 **62** can be synthesized using a [5+3] glycosylation of pentasaccharide donor **55** and trisaccharide acceptor **15** under NIS, TMSOTf activation conditions. The protected octasaccharide **61** can then be subjected to global deprotection to afford completely deprotected octasaccharide **62** or two repeating unit fragment of PS A1 (Scheme 15).




**Scheme 15:** Synthesis of two repeating unit fragment of PS A1 **62**

# Chapter 4

# Development of Novel Microwave Labile Protecting Groups Through Electron Tuning of Benzyl Protecting group

## 4.1. Introduction:

The importance of protecting groups in organic synthesis is very well known to synthesize very complex molecules through selective blocking or protection of reactive functional groups like amine, hydroxyl, thiol and others.[124] Even though many reports in the literature mention ruling out the use of protecting groups[139] in the synthesis of complex natural products in the context of sustainable chemistry,[140] yet many complex natural product syntheses require the installation and removal of the protecting groups to obtain the specific chemical linkages.[124, 141] Carbohydrate chemistry deals with the synthesis of complex oligosaccharides (linear/branched), where simple monosaccharides are linked through stereospecific glycosidic linkages (oxo/thio).[121a] In general, sugars are polyhydroxyl moieties with hydroxy groups arranged in selective *cis*/*trans* relationship to each other depending on the sugar residue (glucose/galactose/mannose). The use of protecting groups in the complex oligosaccharide synthesis is inevitable to access the stereospecific glycosidic linkages.[104, 117, 119, 121c] Hence, retrosynthesis is designed in a way

so that the respective hydroxyl functionalities required in the glycosidic linkage formation are protected with temporary protecting groups and where as other hydroxyl groups are protected with permanent protecting groups, cleaved in the late stage in the process of global deprotection.

So far, a huge number of protecting groups with respect to the functional groups like thiol, amino and hydroxyl groups have been developed in orthogonal/non-orthogonal fashion to ease the process of oligosaccharide assembly.[124, 142] The main limitation of using protecting groups followed by the deprotection as per the synthetic strategy is the addition of two more steps to the overall scheme, reducing the overall yield of the chemical synthesis.[143] The ideal protecting group is supposed to have features like ease of installation/removal, high yields and compatibility with other functional groups.[144] Until now, a variety of protecting groups have been developed, the need for more protecting groups closer to the concept of "ideal protecting group" is still under progress. Based on the mode of deprotection, protecting groups known to date are classified into chemo-labile (CLPG)[124] and photo-labile (PLPG).[145] In the category of chemo-labile PGs, a huge number of PG are available that can selectively installed and removed under specific conditions. The ester PGs (OAc, OBz, OLev and others) cleavable under basic conditions, benzyl/related PGs (OBn, OPMB and others) cleavable under reductive or oxidative conditions, carbamate PGs (NHFmoc, NHCbz, NHTroc and others) cleavable under reductive or basic conditions and silyl PGs (OTBS, OTBDPS, OTES and others) cleavable using fluoride source are the most commonly employed groups in the chemical synthesis of variety of natural products and oligosaccharides (Figure 15).[124] Until now, a variety of

photo-labile protecting groups have been developed and used in the chemical synthesis, drug development, material science and others (Figure 16).[146]

Among the various available CLPGs, benzyl PGs are of very good importance, due to their stability to a wide variety of chemical modifications and hence are used as permanent PGs in the oligosaccharide assembly.[124] Various types of benzyl (Bn) PGs have been developed based on the principle of electron tuning (Figure 15),[147] in order to access benzyl PGs that are orthogonal to each other, for example, *p*-methoxy benzyl group (PMB) can be cleaved orthogonally in the presence of benzyl (Bn) group oxidatively using DDQ and with Lewis/Bronsted acids.[124]

The above mentioned deprotection protocols require the use of reagents in stoichiometric quantities, lead to tedious workup procedures in some cases and purification of the desired compound from the by-products.[148] Hence, the requirement of novel protecting groups with the ease of installation and deprotection under mild conditions are of high importance matching the requirements for an ideal protecting group.

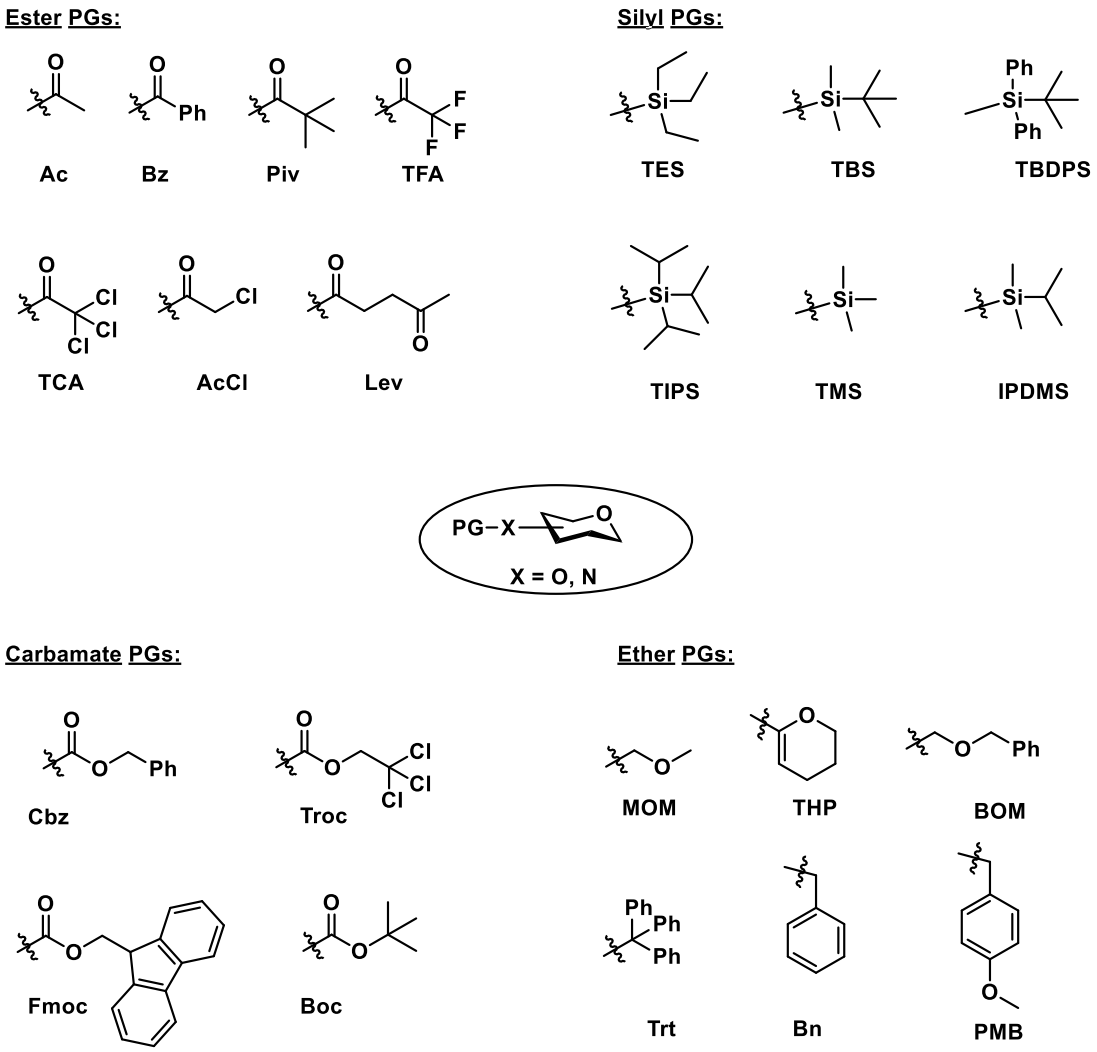
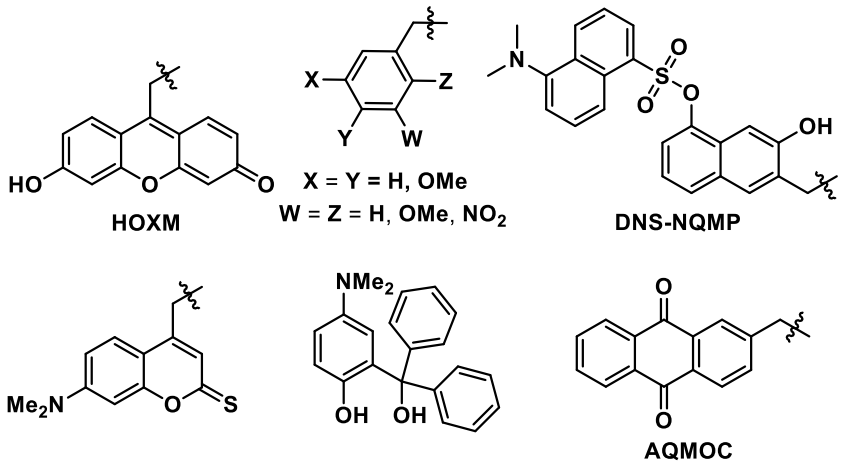



**Figure 4 - 1:** Commonly employed PGs in carbohydrate chemistry

**Photo-labile protecting groups:**




**Benzyl PGs based on electron tuning:**

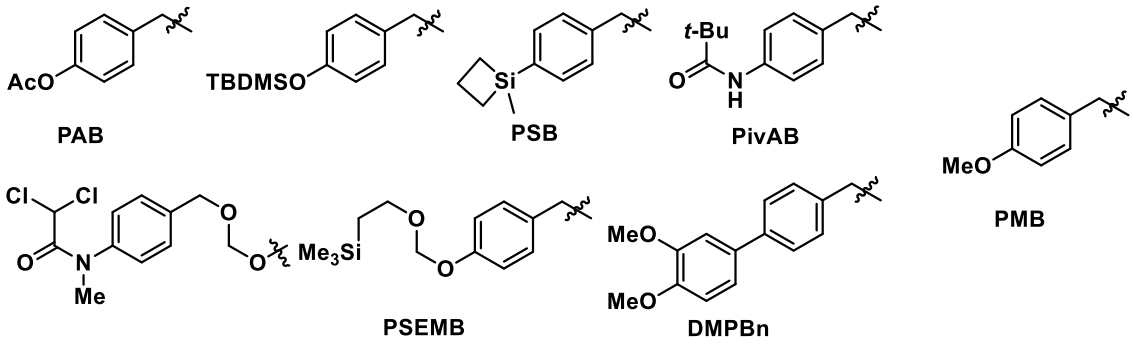



**Figure 4 - 2:** Photo-labile protecting groups (PLPGs) and benzyl-based new PGs

The new set of para substituted *N*,*N*-dialkyl amino benzyl protecting groups (PDMAB or PDEAB) were developed using the principle of electron tuning, can be deprotected under neutral conditions with protic solvent like methanol under microwave irradiation. The PDMAB/PDEAB protecting groups are very electron rich and can be cleaved with Lewis or Bronsted acids. The deprotection protocol under microwave

irradiation is compatible with various other PGs like OAc. OBz, Bn, silyl and even PMB group (Figure 17).

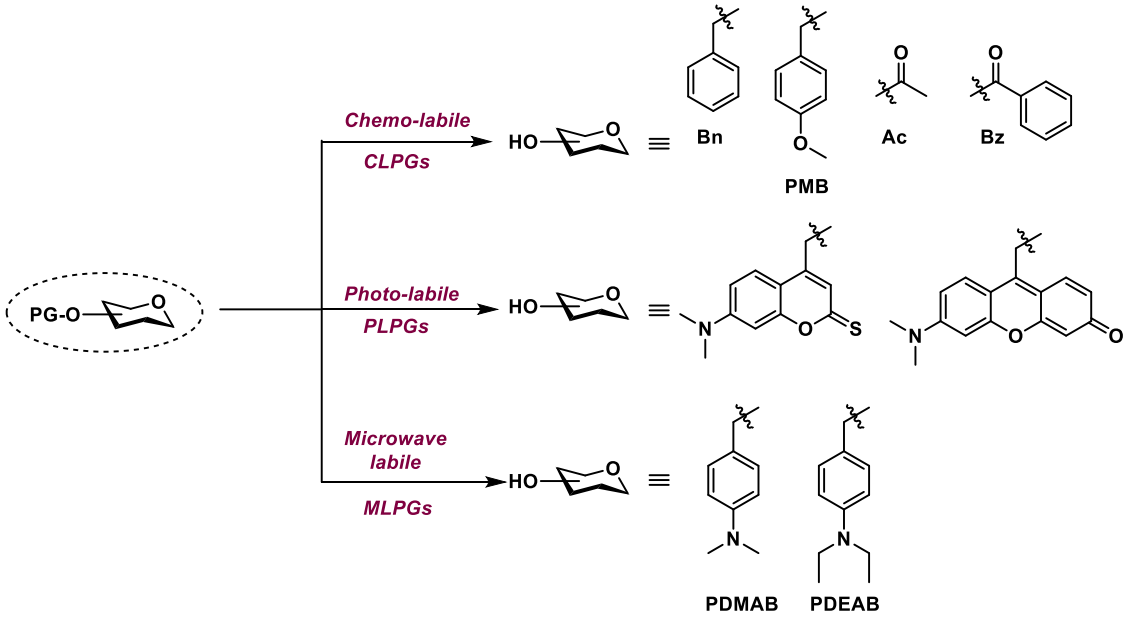
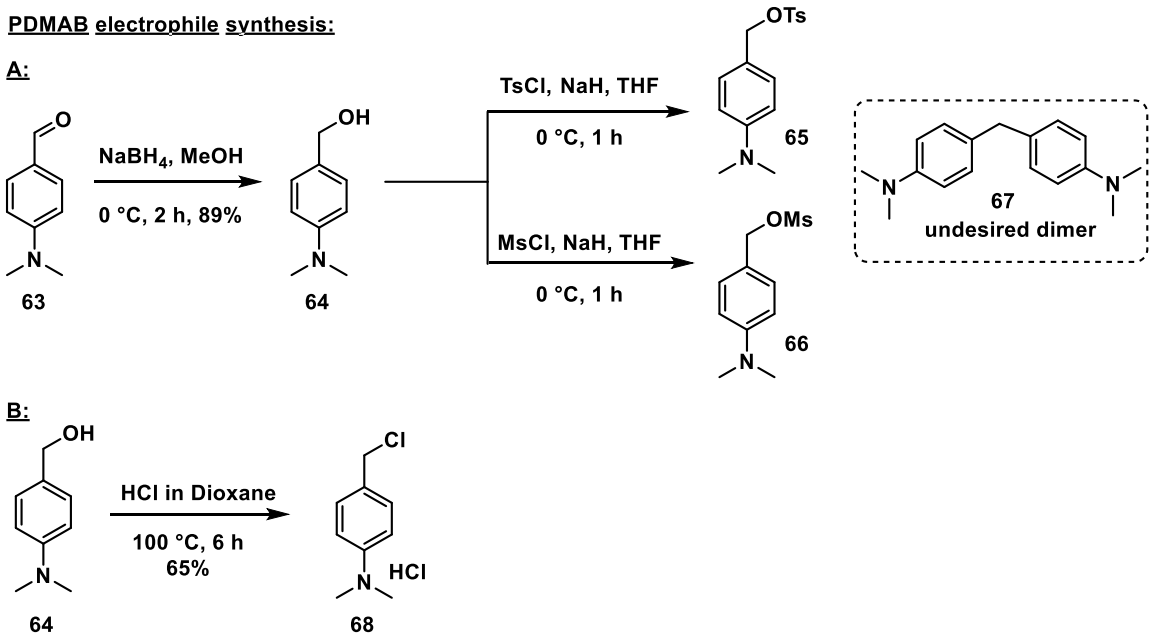



**Figure 4 - 3:** Chemo, photo and microwave-labile PGs

## 4.2. Microwave-labile protecting groups (MLPGs):

### 4.2.1. Installation of PDMAB/PDEAB protecting groups

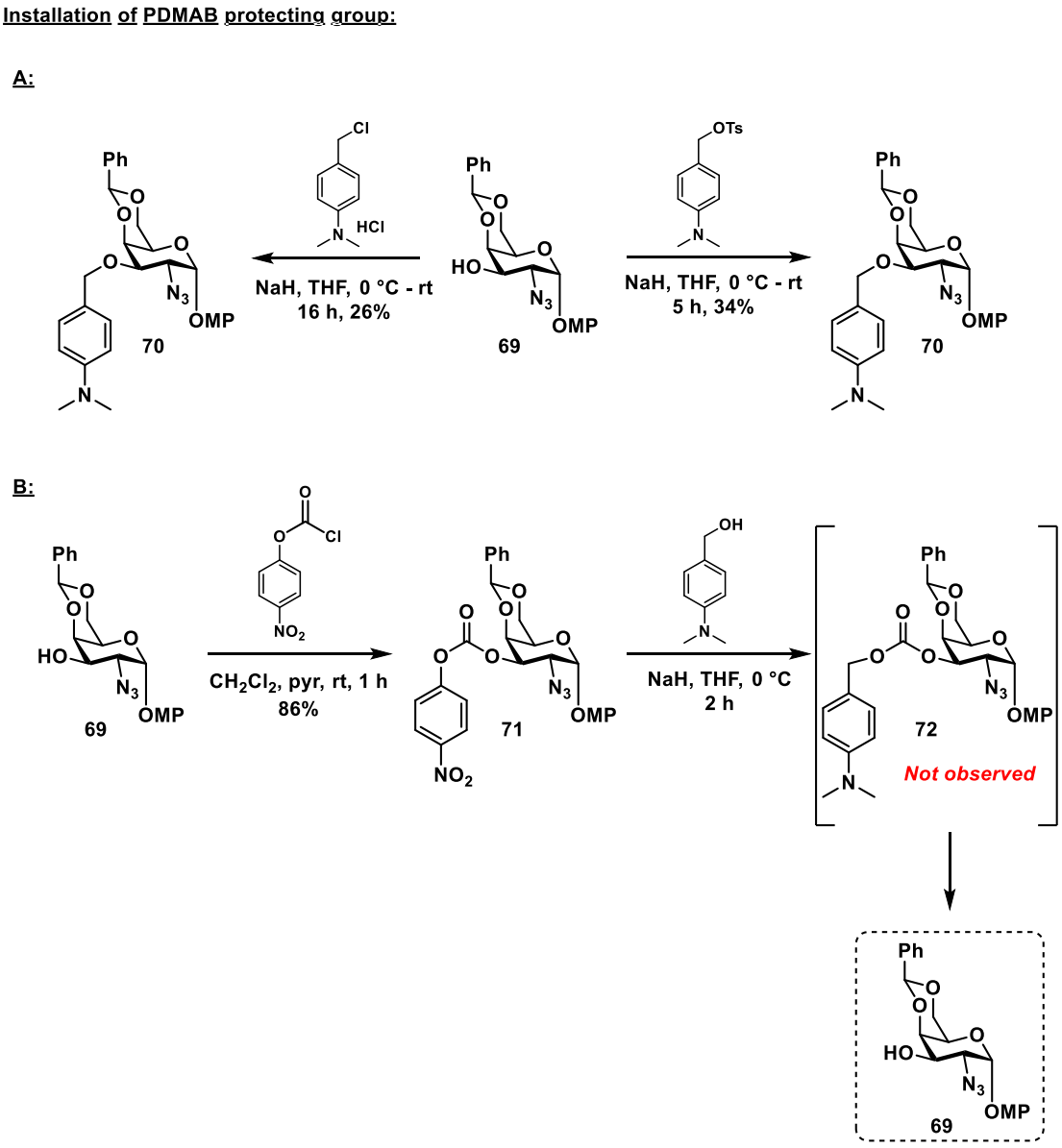
Installation of the PDMAB protecting group started with the synthesis of suitable electrophilic source of PDMAB group. The commercially available *p*-*N*,*N*-dimethyl benzaldehyde **63** was reduced to the corresponding alcohol **64** using $NaBH_4$ in 89% yield,[149] followed by treatment with TsCl and MsCl, to convert the corresponding hydroxyl into a good leaving group (Scheme 16, A). The OMs/OTs of PDMAB **65**/**66** were unstable, decomposed readily during the process of isolation and lead to the formation of the dimer **67**. The PDMAB tosylate **65** was used directly from the reaction mixture.




**Scheme 16:** Synthesis of PDMAB electrophiles

The chloro derivative of PDMAB **68** was also synthesized from the alcohol **64** using HCl in dioxane (Scheme 16, B). With compounds **65** and **68** in hand, installation of PDMAB group was attempted. The alcohol **69** was used with OTs of PDMAB **65** in THF using NaH as a base to obtain the desired PDMAB protected sugar **70**, the reaction was low yielding due to the decomposition of the PDMAB tosylate under the reaction conditions. Similarly, the PDMAB chloride salt **68** was used under the same conditions, the desired compound **70** was obtained in lower yields due to the instability of PDMAB chloride under basic conditions and conversion to the undesired dimer side product **67** (Scheme 17). The synthesis of PDMAB protected carbonate sugar **72** was attempted *via* *p*-nitrophenyl carbonate **71** through nucleophilic substitution of *p*-nitrophenyl group under basic conditions. The substitution of *p*-nitrophenyl group was confirmed by the bright yellow color of the *p*-nitrophenolate anion under basic conditions, but the *in situ* formed

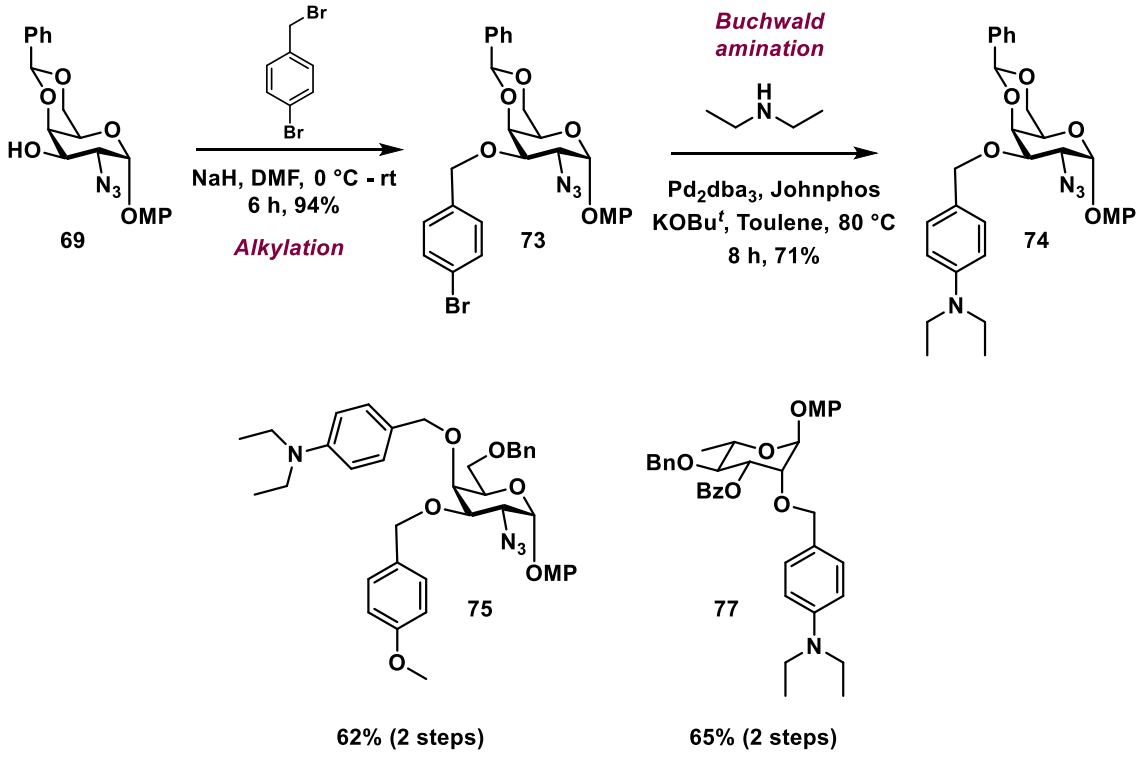
PDMAB carbonate decomposed through decarboxylation process to give back the corresponding alcohol starting material **69** (Scheme 17).




**Scheme 17:** Installation of PDMAB protecting group

Plante *et. al* developed a protocol to install para monosubstituted or disubstituted amino benzyl functionalities using alkylation and Buchwald coupling strategy.[150] Using the two-step protocol, the installation of PDMAB protecting group was attempted.

**Installation of PDMAB protecting group:**

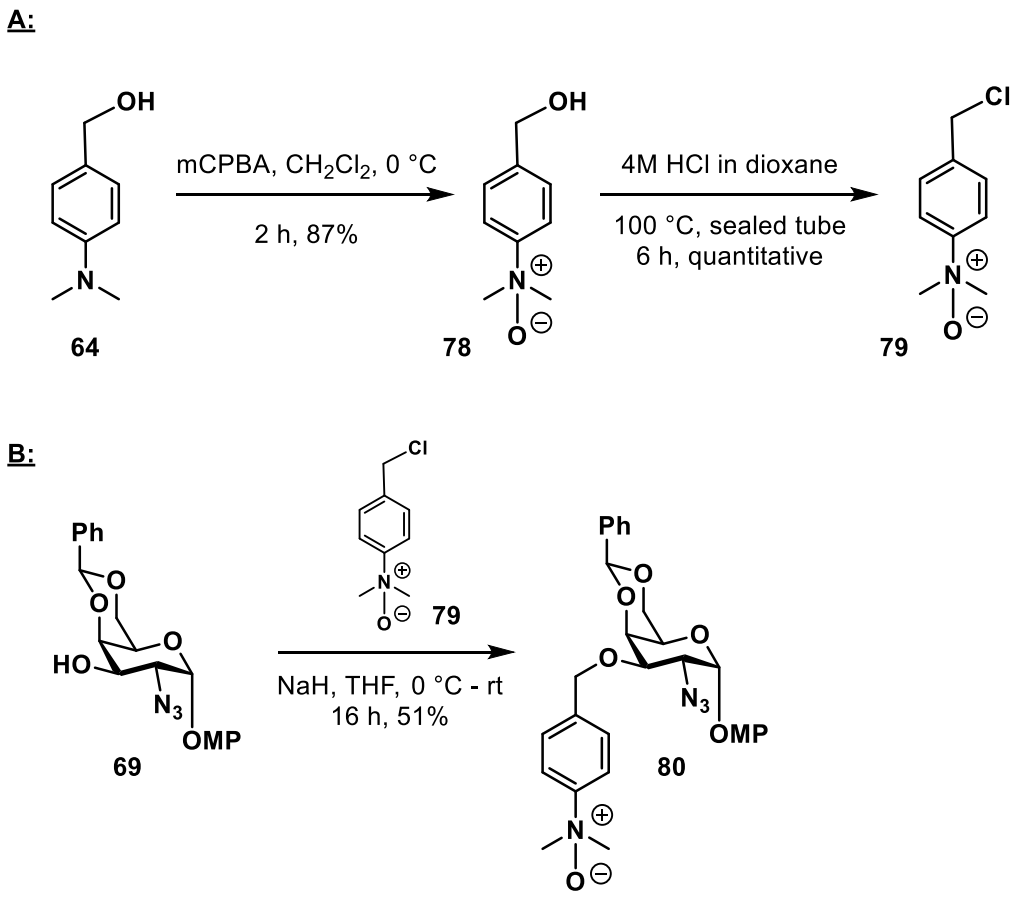



**Scheme 18:** Installation of PDMAB protecting group

Alcohol **69** was alkylated using *p*-bromo benzyl bromide to obtain the desired bromo compound **73** in 94% yield, followed by the palladium catalyzed Buchwald amination afforded the desired PDEAB protected compound **74** in 71% yield. Using the same strategy three other PDEAB derivatives (**75**, **76** and **77**) were synthesized (Scheme 18).

### 4.2.2. *N*-oxide based PDMAB protecting group

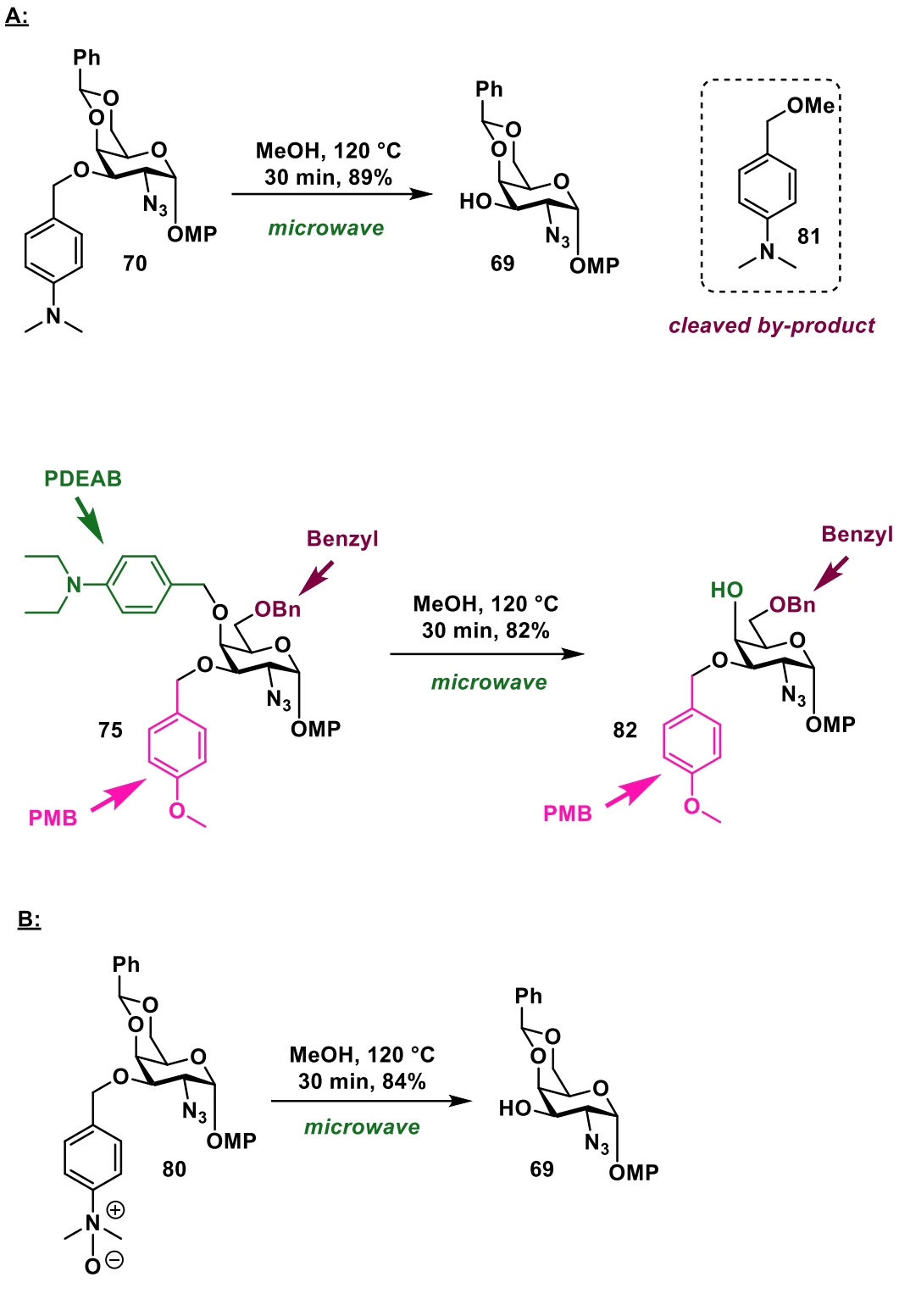
Due to the unstable nature of the PDMAB/PDEAB OTs, OMs and chloro derivatives, use of *N*-oxide based PDMAB derivatives as electrophiles was attempted.




**Scheme 19:** Installation of PDMAB *N*-oxide protecting group

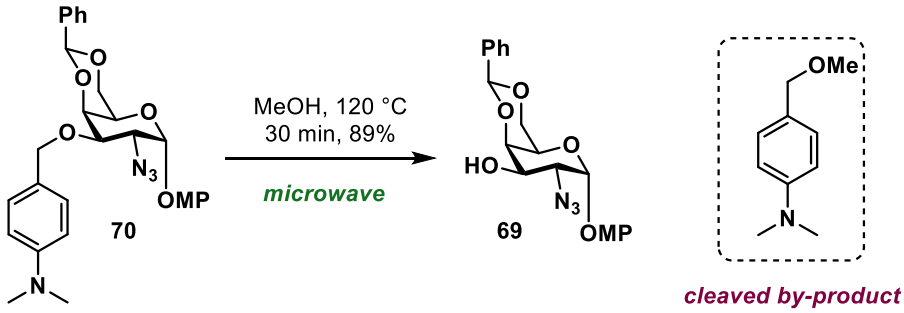
The PDMAB alcohol **64** was oxidized using mCPBA into the corresponding *N*-oxide alcohol **78** in 87% yield, followed by treating with 4M HCl in dioxane to obtain the desired chloro *N*-oxide compound **79** in quantitative yield. The alcohol **69** was treated with NaH in THF for 15 min, followed by the addition of chloro *N*-oxide **79** afforded the desired PDMAB *N*-oxide protected sugar **80** in 51% yield (Scheme 19).

### 4.2.3. Deprotection of PDMAB/PDEAB under neutral conditions:

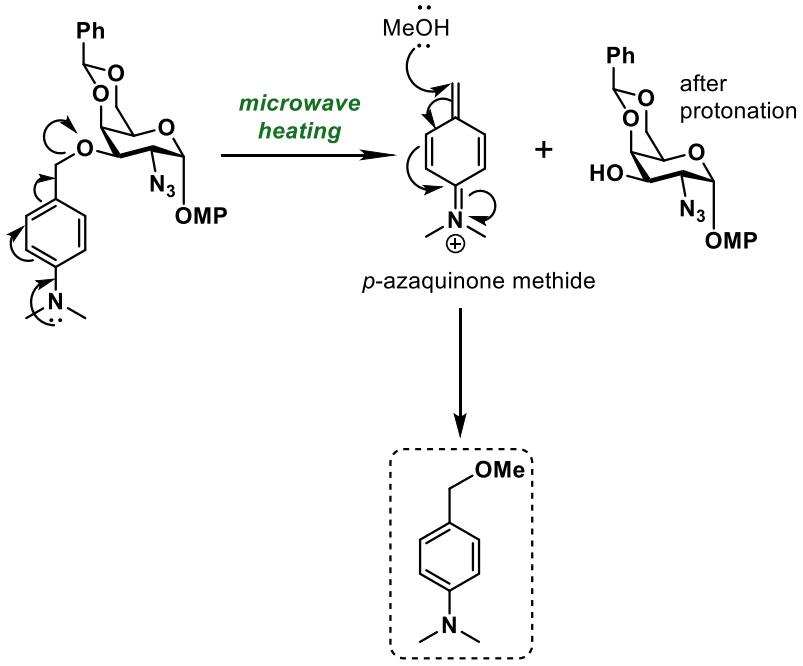



**Scheme 20:** Deprotection of PDEAB and PDMAB *N*-oxide protecting groups

With both PDMAB/PDEAB, PDMAB *N*-oxide protected sugars in hand, deprotection of the respective protecting groups was attempted. The compound **70** was dissolved in methanol and subjected to microwave irradiation at 120 ˚C for 30 min. The deprotection proceeded very smooth affording the desired alcohol **69** in 89% yield (Scheme 20).




**Proposed mechanism:**

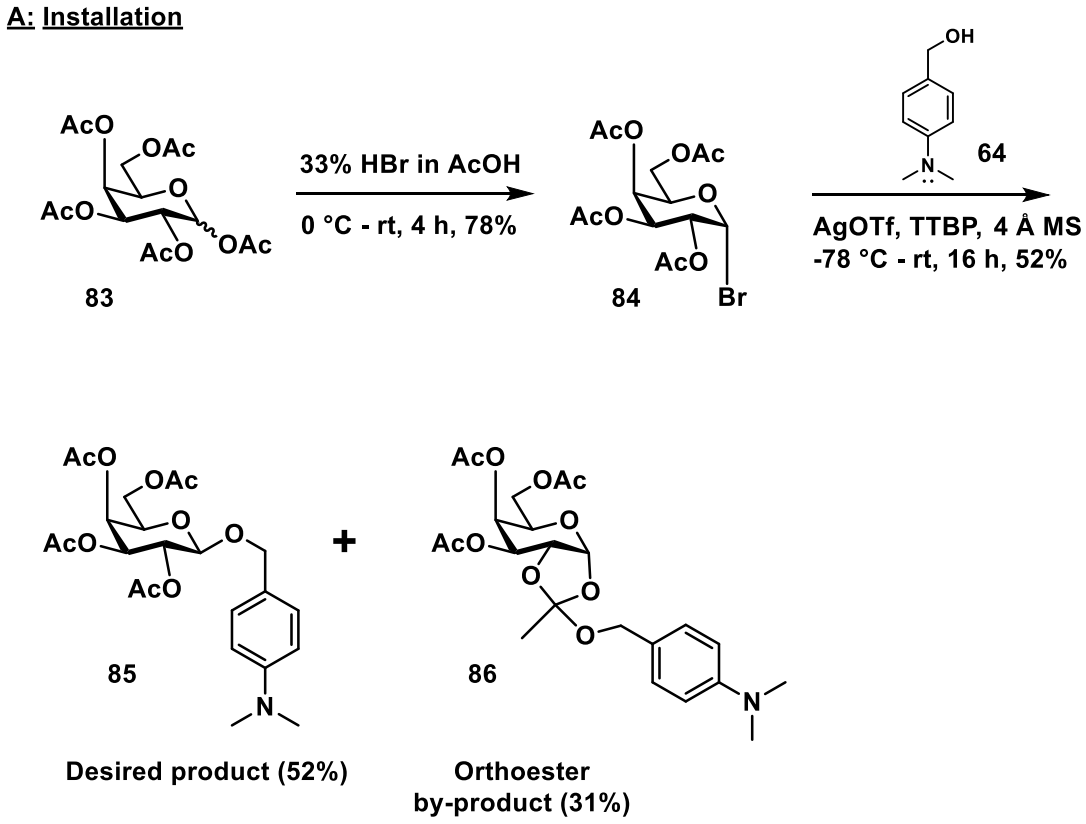


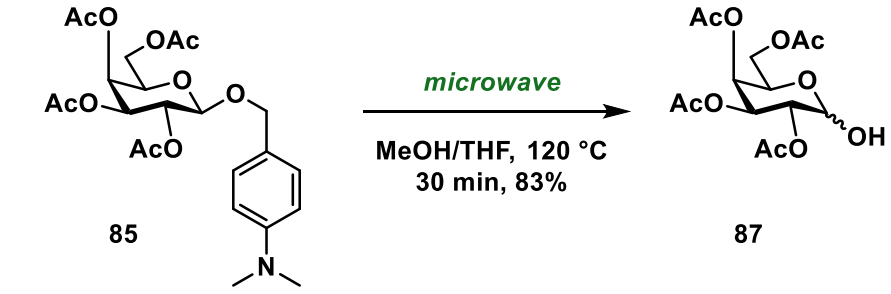

**Scheme 21:** Proposed mechanism for PDMAB/PDEAB group cleavage

In order to test the orthogonality of the PDMAB/PDEAB protecting groups with respect to the Bn, PMB PGs, and compound **75** was subjected to microwave irradiation at 120 ˚C for 30 min. The selective cleavage of PDEAB group in the presence of PMB and Bn PGs was observed (Scheme 20) in 82% yield. The *N*-oxide protected sugar **80** was also subjected to the similar microwave deprotection protocol, to our surprise, deprotection was successful in 84% yield (Scheme **20**). Based on the undesired dimer **67** formation due to the instability of PDMAB OTs, OMs and chloro derivatives and based on the formation of *O*-methyl PDMAB by-product **81**, we propose a plausible mechanism proceeding probably through *p*-azaquinone methide intermediate in an $S_N1$ pathway (Scheme 21).

### 4.2.4. Installation and deprotection of PDMAB at anomeric position:

With the protocol optimized for the protection and deprotection of PDMAB group onto the free hydroxyl groups, the installation of PDMAB group at the anomeric position was attempted. The compound **83** was converted to bromo derivative **84** using HBr in AcOH at 0 ºC to room temperature in 78% yield. The respective bromo derivative **84** was subjected to glycosylation with PDMAB alcohol **64** using AgOTf at -78 ºC to room temperature over a period of 16h to afford the desired anomeric PDMAB compound **85** in 52% yield and the undesired orthoester by-product (31% yield) was also observed. The respective anomeric PDMAB derivative **85** has the potential to be used as glycosyl donor, as microwave irradiation will lead to the formation of anomeric alkoxide intermediate that can be coupled with various electrophiles like OTs, OMs and OTf bearing monosaccharides through *O*-alkylation strategy. The deprotection was attempted in an aprotic solvent like tetrahydrofuran (THF) and the hemiacetal formation was observed in 83% yield (Scheme 22).




**Scheme 22:** Anomeric PDMAB installation and deprotection

## 4.3. Future directions:

Using the *O*-alkylation strategy, the anomeric PDMAB derivatives can serve as glycosyl donors upon microwave irradiation and can be coupled with electrophilic monosaccharide derivatives and hence open new platform for the oligosaccharide assembly. The most prominent feature of using PDMAB donors is that the glycosylation reaction can be accomplished under neutral conditions without any activators/promoters. Hence the novel concept of *neutral glycosylation*, coupled with chiral solvents like (R/S) 2-methyl THF or with chiral additives can lead to the development of a new methodology accessing stereoselective glycosidic linkages under neutral conditions.

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