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## 9.05 Technology-Enabled Synthesis of Carbohydrates

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### Glossary

**Anchimeric effect**  
The interaction of a reaction center with a lone pair of electrons from another atom or the electrons of a sigma or pi bond; the anchimeric effect is also known as neighboring group participation.

**Anomeric effect**  
A stereoelectronic effect that describes the tendency of heteroatomic substituents adjacent to a heteroatom within a cyclohexane ring to prefer the axial orientation instead of the less hindered equatorial orientation.

**Glycoconjugate**  
Mono-, di-, or oligosaccharide that is covalently bound to a molecule, often a peptide, protein, or lipid.

**Glycosidic bond**  
Covalent bond formed between the hemiacetal group of a mono-, di-, or oligosaccharide and the hydroxyl group of another molecule.

**Glycosyl acceptor**  
Mono-, di-, or oligosaccharide that serves as a nucleophile in a glycosylation reaction.

**Glycosyl donor**  
Mono-, di-, or oligosaccharide that serves as an electrophile in a glycosylation reaction; the glycosyl donor generally determines the nature of the glycosidic linkage.

**Glycosylation reaction**  
Reaction between glycosyl donor and a glycosyl acceptor that results in a glycosidic bond.

**Monosaccharide**  
A single sugar residue.

**Oligosaccharide**  
Multiple sugar residues connected via glycosidic bonds.
9.05.1 Introduction

Nucleic acids, proteins, and glycoconjugates play critical roles in a number of important signal transduction pathways. The development of rapid and efficient automated platforms for the synthesis of nucleic acids and peptides from their monomeric building blocks (Figure 1 (a) and (b)) has led to a better understanding of the fundamental interactions these biomolecules play in important biological processes. As a result, the fields of genomics and proteomics have grown exponentially with the development of new therapeutic strategies for disease prevention and treatment. Until recently, the lack of a general and accessible approach for the construction of oligosaccharides meant that only specialized research laboratories were capable of accessing defined glycoconjugates. This has slowed the pace of research in this area and in turn, our understanding of the diverse roles of these compounds in nature.

The major challenge with developing a general and accessible approach for the synthesis of oligosaccharides is inherent in the reaction products. The linear phosphodiester and amide bond linkages required for nucleic acid and peptide synthesis are easier to generate because the individual building blocks lack significant steric hindrance and the newly formed bonds are nonstereogenic. In contrast, the synthesis of oligosaccharides, which can be linear or branched, often involves more sterically encumbered building blocks and the newly formed bond is part of a stereogenic center. In order to be useful the reaction must generate the desired linkage with high selectivity.

The development of a first-generation automated solid-phase oligosaccharide synthesizer by Seeberger and coworkers \(^1\) led to the first reported automated synthesis of three linear 1,2-α-linked mannan oligosaccharides and a branched phytoalexin elicitor β-glucan (Figure 2). The synthesis of these glycoconjugates, which play important roles in the immune response to pathogenic fungi such as \textit{Candida albicans}, \(^2\) began what would become a new chapter in the preparation of glycoconjugates for biological evaluation. Since then, automation has proven to be vastly beneficial. Additional bacterial antigens, cancer antigens, vaccine...
candidates, and N-linked core oligosaccharides are just a few of the glycoconjugates that have been rapidly synthesized using automation. Moreover, these glycoconjugates have been synthesized on scales that would have been impossible using standard solution-phase synthetic techniques.

The goal of this chapter is to review the historical aspects of solid-phase oligosaccharide synthesis as well as recent developments including engineering advancements that have recently led to the design of a fully automated platform, and new and improved chemistries that have broadened the scope of the reactions that can be used in automation. The automated solid-phase synthesis of several carbohydrate constructs including bacterial and viral antigens, cancer antigens, vaccine candidates, and N-linked core oligosaccharides is also presented.

9.05.2 Principles of Automated Oligosaccharide Synthesis

Automated solid-phase oligosaccharide synthesis uses an automated synthesizer to make glycosidic linkages in an analogous process to the way DNA synthesizers generate phosphodiester linkages and peptide synthesizers generate peptide linkages. The general process is illustrated in Figure 3. Selectively functionalized monosaccharide building blocks containing temporary and permanent protecting groups (commonly referred to as donors) serve as electrophiles and are added sequentially to a growing oligosaccharide chain, which serves as a nucleophile (or acceptor). The acceptor is connected via an inert linker to a solid support that is generally insoluble in organic solvents. Each glycosidic linkage is made by activating the appropriately functionalized monosaccharide building block (donor) in the presence of the growing nucleophilic oligosaccharide (acceptor) to couple to the two molecules. After each glycosylation reaction, the growing oligosaccharide is washed, the appropriate temporary protecting groups are removed to expose the next functional group for coupling, and the process is repeated until the desired oligosaccharide has been synthesized. The oligosaccharide is then removed from the solid support, purified if necessary, and the remaining temporary and permanent protecting groups are removed. The oligosaccharide can then be functionalized for further study.

The key features of the automated process, shown in Figure 4, include the automation platform, an insoluble solid support, an inert linker, and selectively functionalized carbohydrate building blocks containing temporary and permanent protecting groups. Developments in each of these areas have had a significant impact on the evolution of automated solid-phase oligosaccharide synthesis.

9.05.3 Automation Platforms

9.05.3.1 First-Generation Automated Solid-Phase Oligosaccharide Synthesizer: Modified ABI 433 Peptide Synthesizer for Automated Oligosaccharide Synthesis

The first automated synthetic platform for automated solid-phase oligosaccharide synthesis was developed by Seeberger and coworkers and based on a modified Applied Biosystems peptide synthesizer, the ABI 433. This system was chosen as a prototype because it was affordable and commercially available, it could accommodate up to nine different reagents/building blocks at one time, and required minimal modifications, namely a custom made jacketed reaction vessel connected to a variable cryostat to allow for controlled cooling of the reaction mixture.

As a prototype, the ABI synthesizer played a critical role in the development of automated oligosaccharide synthesis. The synthesizer was reliable, and solvent delivery and waste removal were relatively efficient. In addition, reaction mixing, which was
Figure 3 Automated solid-phase oligosaccharide synthesis.
achieved by agitation of the reaction vessel, was effective in producing a uniform heterogeneous solution. However, the instrument, which was designed for peptide synthesis, had several shortcomings. Reagent delivery, which was achieved via solenoid valves, was imprecise. Therefore, the delivery of small reagent volumes or the delivery of reagents at a controlled delivery rate was difficult to achieve. In addition, the system could not be modified to accommodate additional reagents limiting the number of different glycosylation reactions that could be performed in a single run. Finally, temperature control required manual adjustment of the cryostat therefore, necessitating the continuous presence of an operator. For these reasons, the Seeberger group focused their efforts on the development of a new fully automated solid-phase oligosaccharide synthesizer.

9.05.3.2 Second-Generation Automated Solid-Phase Oligosaccharide Synthesizer: The First Fully Automated Oligosaccharide Synthesizer

In order to provide a more efficient platform for automated solid-phase oligosaccharide synthesis, Seeberger and coworkers collaborated with Ancora Pharmaceuticals to develop the first fully automated solid-phase oligosaccharide synthesizer. The new design, introduced in 2012, addressed the shortcomings of the modified ABI 433 peptide synthesizer. Syringe pump technology for precise reagent delivery was combined with solenoid valves for rapid pressure-driven washing steps. The system also included 16 vessels capable of delivering up to 16 reagents building blocks at one time. In addition, a mechanical controller was designed to serve as an interface between the instrument and a personal computer, which provided full control of the system including temperature modulation. The instrument is capable of reaching temperatures as low as \(-50\, ^\circ \text{C}\) via adjustable cryostat and as high as \(90\, ^\circ \text{C}\) via heating block.

9.05.3.3 HPLC-Based Automated Oligosaccharide Synthesizer Platform

Demchenko and co-workers recently reported on the first modified HPLC for automated solid-phase oligosaccharide synthesis. Their experimental setup, which includes an unmodified three-headed pump HPLC system and an onboard variable range UV detector, uses a standard chromatography column packed with preswelled polymer resin. The benefit of this system is that it employs a commercially available HPLC found in almost any synthetic or analytical laboratory, and the programming can be accomplished using standard HPLC operating software.

Demchenko’s HPLC-based solid-phase oligosaccharide synthesizer is the first automated system that uses real-time UV-Vis reaction monitoring and shows significant promise as an emerging technology. However, one significant disadvantage is the limited number of reagents/building blocks the system can accommodate without significant modification or the continuous presence of an operator.

9.05.4 Solid Support

Commercially available nonsoluble polystyrene solid supports such as Merrifield resin and TentaGel (Figure 5) are most commonly employed in automated solid-phase oligosaccharide synthesis. These resins generally exhibit high loading, are chemically inert to most of the reaction conditions required for glycosylation, and can be easily filtered from the reaction solution. The only disadvantage is that they require swelling to expose the reactive sites of the resin. Incomplete swelling can lead to incomplete reactions and deletion sequences. Controlled pore glass (CPG), which is a rigid, mechanically robust material that does not shrink or swell in the presence of solvents, has been used with high success for the synthesis of oligonucleotides; however, it has not attracted much attention for automated oligosaccharides synthesis for two major reasons. First, CPG loading, which is
Second, the growing oligosaccharide has been shown to eventually block the glass pores reducing diffusion of the reagents to the reactive sites. CPG with larger pore sizes has been used with some success, although the resin tends to be significantly more fragile.

### 9.05.4.1 Merrifield Resin

The majority of the automated solid-phase oligosaccharide syntheses reported to date have made use of Merrifield resin, a chloromethylated polystyrene resin that has been widely employed in peptide synthesis. In addition to high loading, Merrifield resin is relatively inert under the acidic and basic conditions used in the automation process. One significant disadvantage in comparison to the CPG used in DNA synthesis is the number of washing steps required to completely remove excess reagents and byproducts from the porous polystyrene matrix. In addition, the reactive sites of the resin are only exposed in the presence of a swelling solvents such as dichloromethane, tetrahydrofuran, and toluene, prohibiting the use of nonswelling solvents such as hexane and methanol.

### 9.05.4.2 Tentagel

TentaGel has also been used in the automated solid-phase synthesis of oligosaccharides. TentaGel consists of polyethylene glycol cross-linked to polystyrene via an ether linkage. Tentagel combines the benefits of a soluble polyethylene glycol support with the insolubility and handling characteristics of a polystyrene bead. TentaGel also displays relatively uniform swelling in a variety of solvents from medium to high polarity ranging from toluene to water. One significant disadvantage in comparison to Merrifield resin is that the hydrophilic nature of the resin can make the removal of residual water difficult, leading to reduced yields during glycosylation.

### 9.05.5 Linker

The linker connects the first unit of the growing oligosaccharide chain to the solid support. To be effective the linker must remain intact during synthesis, and be selectively cleavable under relatively mild conditions at the end of the synthesis. Optimally, the cleaved linker should have a functional handle for attachment to a protein carrier, chip, or other surface. To date, four linkers have been commonly employed in automated oligosaccharide synthesis (Figure 6): alkene-diol (octenediol or butenediol) linkers, a bifunctional linker, and a succinamic linker.
9.05.5.1 Alkene–Diol Linkers

Octenediol and butenediol linkers, which contain a single double bond, fit the requirements for a suitable linker for automated solid-phase oligosaccharide synthesis. These linkers are generally connected to the solid support via an ether or ester linkage. After synthesis, the linker is cleaved by cross-metathesis (or base hydrolysis then cross-metathesis) using Grubbs’ first-generation catalyst (Grubbs’ I) in the presence of ethylene. The resulting fully protected n-pentenyl glycoside (octenediol) or allyl glycoside (butenediol) can be hydrolyzed to the free reducing sugar or activated and then glycosylated. The carbon–carbon double bond can also be modified to produce a handle with a nucleophile suitable for conjugation.

There are three major disadvantages to the use of the octenediol and butenediol linkers. The first is that the alkene prohibits the use of electrophiles, such as those required for the activation of thioglycosides, during the automated process. The second is that the Grubbs’ catalyst required for cleavage from the resin is expensive and often difficult to remove from the final reaction products. Finally, the alkene handle requires further functionalization to be useful for conjugation and biological evaluation; such transformations can reduce the overall yield of the final oligosaccharide product.

9.05.5.2 Bifunctional Linker

The bifunctional linker was prepared to address the issues associated with the octenediol and butenediol linkers. The bifunctional linker, which is connected to the solid support via an ester linkage, is fully compatible with most common glycosylation conditions, including the electrophilic conditions required for the activation of thioglycosides. Cleavage of the ester using base-catalyzed hydrolysis is inexpensive and accomplished under mild conditions. After purification, hydrogenolysis of the remaining carbamate reveals a C5 linker with a terminal amine suitable for conjugation. The main disadvantage with the bifunctional linker is that it’s use limits the types of temporary protecting groups that can be used; acetate groups can no longer serve as temporary protecting groups since the reaction conditions required to remove them would also result in the cleavage of the oligosaccharide from the resin.

9.05.5.3 Succinamic Linker

A succinamic linker was recently reported for use in automated solid-phase oligosaccharide synthesis. This linker, which is connected to the solid support (TentaGel) via an amide linkage, can also be cleaved using base-catalyzed hydrolysis. The succinamic linker offers similar advantages to the bifunctional linker, and in principle could support the electrophilic conditions required for thioglycoside activation. The main disadvantage with the succinamic linker is that the alcohol handle produced on hydrolysis from the resin must be further functionalized for conjugation.

9.05.6 Building Blocks

The identification and synthesis of the building blocks required to construct the oligosaccharides is the most laborious and time-consuming task of the automated process. Protecting group patterns must be designed to achieve the desired connectivity and stereoselectivity in high yield. In addition, the nature of the protecting group can directly influence the reactivity of the building block, affecting reaction times and yield.

The basic features of a building block are highlighted in Figure 7 and include the anomeric leaving group (LG), a participating group (for 1,2-trans linkages) or nonparticipating group (for 1,2-cis linkages) at the C2 position (R1), temporary protecting groups that are stable under glycosylation conditions but readily removed for the next glycosylation reaction (R2) and permanent protecting groups that remain intact until the final global deprotection step (R3).

Mammalian carbohydrates are commonly comprised of ten monosaccharides: glucose, galactose, mannose, sialic acid, N-acetylgalactosamine, N-acetylglucosamine, fucose, glucuronic acid, iduronic acid, and xylose (Figure 8). In principle, each carbohydrate residue can be linked through any of its different functional groups and each linkage generates a new stereocenter. The potential diversity is significant. For example, the number of theoretical structures possible for a hexasaccharide comprised from any one of the ten mammalian monosaccharides is approximately 190 billion!

It has been estimated that 224 building blocks would be required to assemble any given mammalian oligosaccharide. However, recent studies have suggested that only 36 building blocks would be required to access approximately 75% of all known mammalian oligosaccharides due to significant overlaps in common connectivities. Thirty of these building blocks are shown in Figure 9 and are ranked by relative abundance in mammalian oligosaccharides.

The type II Lewis blood group oligosaccharide Le°–Le° (nonasaccharide) provides a good example of the number and types of carbohydrate linkages that can be made. As illustrated in Figure 10, the Le°–Le° molecule is comprised of four β-linked sugar residues: glucose, galactose, N-acetylgalactosamine, and fucose. Individual differences in the connectivities of these four sugars require that five differentially functionalized building blocks from Figure 10 would be required to synthesize Le°–Le° molecule.
9.05.6.1 Anomeric Leaving Group

In automated solid-phase oligosaccharide synthesis, the mono- or disaccharide with the LG, which serves as a glycosyl donor or electrophile, is activated in the presence of a nucleophile. There are three common LGs employed in automated solid-phase oligosaccharide synthesis (Figure 8): phosphates, imidates, and thiols. Historically, glycosyl phosphates and imidates have played the most important roles in automated solid-phase oligosaccharide synthesis. However, a number of automated solid-phase oligosaccharide syntheses employing thioglycosides have been reported recently in the literature.

The accepted mechanism for glycosidic bond formation using glycosyl phosphates, imidates, and thiols under standard glycosylation conditions is illustrated in Scheme 1. Nucleophilic displacement ($S_{N}1$) results in a glycosidic bond with the C2 position playing a role in the resulting stereochemistry of the products. For this reason, the stereochemistry of the LG generally has little impact on the stereochemical outcome of the reaction. However, it has been demonstrated that in some cases anomers may have different chemical properties resulting in enhanced or reduced reactivity.

Glycosyl phosphates can be synthesized from lactols, glycosyl bromides, acetates, trichloracetimidates, nitrates, thioglycosides, 4-pentenyl and 2-buten-2-yl glycosides, glycals, 1,2-orthoesters, and oxazolines. Dibutylphosphate is most commonly employed in automated solid-phase oligosaccharide synthesis, and it is generally activated with a stoichiometric amount of a Lewis acid, most often trimethylsilyl trifluoromethanesulfonate (TMSOTf), at temperatures approximately $-15 \, ^\circ\text{C}$. Glycosyl phosphates and imidates have been shown to be reasonably stable for over a year at $-20 \, ^\circ\text{C}$.

![Figure 7](image-url) Basic features of building blocks used in automated solid-phase oligosaccharide synthesis.
Figure 8  Ten common monosaccharides.
Figure 9  Building blocks required for the synthesis of approximately 75% of all known mammalian oligosaccharides.

Figure 10  Le"⁻⁻Le" (nonasaccharide) monosaccharide diversity.
Scheme 1  General glycosylation strategy.
Scheme 2  Activation conditions for glycosyl phosphates, imidates and thioglycosides.
More recently, thioglycosides have been shown to be effective LGs in automated oligosaccharide synthesis. Thioglycosides can be prepared from a number of sources.\textsuperscript{13} The most common include lactols, glycosyl halides, and peracetylated or 1-acylated sugars. In automated solid-phase oligosaccharide synthesis, thioglycosides are activated using either an \textit{N}-iodosuccinimide (NIS)/TMSOTf or NIS/TfOH promoter system (Scheme 2) at temperatures approximately $-40\,^\circ\text{C}$. The major advantage to using thioglycosides in automated solid-phase oligosaccharide synthesis is their shelf stability. They generally remain stable, even at room temperature, for extended periods. In addition, thioglycosides can be used to access the other donors; phosphates can be accessed directly from thioglycosides, or the thioglycoside can be hydrolyzed to the lactol and then converted to the imidate in two steps.

\subsection*{9.05.6.2 The C2 Position}

The C2 position plays an important role in directing the glycosidic linkage (Scheme 3). Participating groups such as esters, which form an intermediate acyloxonium ion intermediate, create steric hindrance on one face of the donor permitting attack at the opposite face favoring 1,2-\textit{trans} linkages. Nonparticipating groups, such as ethers, are unable to participate and therefore, the intermediate oxocarbenium ion can be attacked from either side of the carbohydrate residue. However, formation of the 1,2-\textit{cis} linkage is often favored due to the anomeric affect.

\begin{center}
\begin{tabular}{|c|}
\hline
\textbf{R1} Neighboring group participation (anchimeric effect) \\
\hline
\end{tabular}
\end{center}

\begin{center}
\begin{tabular}{|c|}
\hline
\textbf{Nonparticipating} \\
\hline
\end{tabular}
\end{center}

\textit{Scheme 3} Effects of the C2 position on glycosylation.

\subsection*{9.05.6.3 Temporary Protecting Groups}

9-Fluorenyl carboxymethyl (Fmoc), levulinoyl (Lev) and silyl-protecting groups (Scheme 4) have proven to be effective temporary protecting groups in automation. The 9-fluorenylcarboxyl group is generally installed using FmocCl in the presence of a pyridine in high yield.\textsuperscript{14} In automated solid-phase oligosaccharide synthesis, the Fmoc group is readily removed by treatment with a solution of piperidine in dimethyl formamide (DMF) to give a fulvene byproduct (which can be used to monitor the progression of the reaction) and the free alcohol, which serves as a nucleophile in the next glycosylation reaction (Scheme 4).
The levulinoyl (Lev) ester group is generally installed using levulinic acid, N,N'-dicyclohexylcarbodiimide (DCC), and dimethylaminopyridine (DMAP) at room temperature. The Lev group is generally removed in automated solid-phase oligosaccharide synthesis using a 0.5 mol l⁻¹ solution of hydrazine in a mixture of pyridine and acetic acid to give the desired alcohol (Scheme 4). These conditions do not affect normal esters such as acetates, benzoates, or pivaloates.

Although less common, silyl-protecting groups are gaining importance in automated solid-phase oligosaccharide synthesis. The most commonly employed silyl-protecting group, tert-butyldimethylsilyl ether (TBS or TBDMS) is commonly installed using TBDMSCl and imidazole in DMF. Removal of the TBDMS group in automation is accomplished using tetrabutyl ammonium fluoride to give the free alcohol (Scheme 4).

### 9.05.6.4 Permanent Protecting Groups

Permanent protecting groups used in automated solid-phase oligosaccharide synthesis include benzyl groups (Bn), pivaloyl (Piv) and benzoyl (Bz) esters, azides (N₃), and N-trichloroacetamides (TCA) groups (Scheme 5). These groups are generally removed after the newly oligosaccharide has been cleaved from the resin and purified.

Benzyl groups (Bn) are the most common permanent protecting groups used in automation due to their stability under relatively acidic and basic conditions. They are generally installed via treatment of the corresponding alcohol with benzyl bromide and sodium hydride in DMF and are removed using standard hydrogenolysis (Scheme 5). Benzyl groups are most commonly reduced to the corresponding benzylamine using tributyltin hydride in refluxing xylene.

Azides (N₃) are generally employed as nonparticipating protecting groups for the synthesis of 2-amino sugars. Azides are commonly introduced by nucleophilic substitution of sugar derived tosylates, mesylates, and epoxides. Azides can also be installed by treating the corresponding amino sugar with sodium azide and triflic anhydride or triflate azide in copper sulfate. Azides are most commonly reduced by hydrogenolysis to the corresponding amine (Scheme 5).

Pivaloyl (Piv), benzoyl (Bz) esters, and N-trichloroacetamides (TCA) are more stable than acetates and do not hydrolyze readily under the standard reaction conditions used for automated solid-phase oligosaccharide synthesis. These groups are generally prepared by treating the free alcohol (Piv or Bz) or amine (TCA) with the corresponding acyl chloride in the presence of a base. Piv and Bz groups are readily hydrolyzed by treatment with strong base. N-Trichloroacetamyl (TCA) groups are generally reduced to the corresponding N-acetyl groups using tributyltin hydride in refluxing xylene.

Acetyl esters (Ac) can serve as temporary or permanent protecting groups depending on the linker chosen for automation. Acetates are most commonly prepared by treatment of the free alcohol with acetic anhydride in pyridine, and they are hydrolyzed using a solution of sodium methoxide in methanol (Zemplén conditions). Octenediol and butenediol linkers are unaffected by these conditions, but the bifunctional and succinamic linkers are readily cleaved. When these linkers are employed, acetate serves as a permanent protecting group.
9.05.6.5 Monosaccharide Reactivity

The nature of the protecting groups can also affect the general reactivity profile of an individual monosaccharide. With regards to glycosyl donors, electron-donating groups stabilize acyloxonium/oxocarbenium ion intermediates by donating electron density to this intermediate via induction. These groups tend to enhance the rate of the glycosylation reaction. In contrast, electron-withdrawing groups destabilize acyloxonium/oxocarbenium ion intermediates by withdrawing electron density from the reactive site. This can have the affect of decreasing the reaction rate, and in extreme cases can impede reactivity altogether. This stabilizing/destabilizing based on electron donation or withdraw is known as the ‘armed-disarmed’ concept, where electron-donating groups ‘arm’ the donor, whereas electron-withdrawing groups ‘disarm’ the donor.33,34,35

When considering the electronics of glycosyl acceptors, electron-donating groups near the nucleophilic hydroxyl group may enhance nucleophilicity. This can lead to faster reaction rates. Electron-withdrawing groups near the nucleophilic hydroxyl group, which remove electron density through induction, can diminish the overall rate of the reaction.

9.05.7 Automated Synthesis of Oligosaccharides

The first automated solid-phase oligosaccharide syntheses reported by Seeberger and co-workers1 began a revolution in the preparation of glycoconjugates for biological evaluation. Since their initial report, automation has been used by scientists around
the world to synthesize more than 30 complex oligosaccharides including bacterial antigens, cancer antigens, vaccine candidates, and N-linked core oligosaccharides. The syntheses of several of these oligosaccharides are presented in Sections 9.05.7.1–9.05.7.10. Examples were chosen for historical purposes as well as to highlight the challenges and complexities of the oligosaccharides prepared using the automation solid-phase oligosaccharide process.

The majority of the oligosaccharides synthesized via automated oligosaccharide syntheses were synthesized using either the first- or second-generation automated solid-phase oligosaccharide synthesizer. In most cases Merrifield resin was used as the solid-support. Glycosylation reactions typically employ a ‘double-coupling’ (Figure 11) or ‘triple coupling’ strategy which involves activating 3 to 5 equivalents of glycosyl donor two or three times with a washing step (to remove unreacted donor and reaction byproducts) in between. This process is employed to ensure complete reaction thereby minimizing deletion sequences which can have a negative impact on the purification of the desired oligosaccharide.

9.05.7.1 α-Mannans and a Phytoalexin β-Glucan

α-Mannan and β-glucans are known to play important roles in the pathogenesis of certain fungi. The first automated solid-phase oligosaccharide syntheses of α-mannan and β-glucan oligosaccharides were reported in 2001 by Seeberger and co-workers using the first-generation automated solid-phase oligosaccharide synthesizer. The syntheses of these compounds provided proof of principle that linear and branched oligosaccharides could be made in automation in high yield using standard LGs such as glycosyl imidates and phosphates.

The synthesis of α-1,2 linked penta-, hepta-, and decα-α-mannans was accomplished using the first-generation solid-phase oligosaccharide synthesizer. Octenediol-functionalized Merrifield resin was repeatedly glycosylated with mannosyl imidate using a double coupling approach (5 equivalents) per cycle in the presence of a catalytic amount of TMSOTf (Scheme 6). The C2 Ac served as both a directing group (for anchimeric assistance) and a temporary protecting group, which could be hydrolyzed under Zemplén conditions. The pentasaccharide (n = 3) was generated in 74% yield demonstrating approximately 95% coupling efficiency at each step. The heptasaccharide (n = 5) and decasaccharide (n = 8) were produced 42% and 34% yields, respectively, demonstrating approximately 90% coupling efficiency at each step.

Two selectively functionalized glucose building blocks were used to generate a phytoalexin elicitor β-glucan (Scheme 7) as the first example of a branching glycan synthesized by automated solid-phase oligosaccharide synthesis. Octenediol-functionalized
Merrifield resin was coupled with two glycosyl phosphate building blocks, monosaccharide \( 2 \), and 1,3-linked disaccharide \( 3 \), containing C2 Piv participating groups. Each iterative ‘double coupling’ required 5 equivalents of the desired phosphate building block (2 or 3) and a stoichiometric amount of TMSOTf. After each glycosylation, the Lev ester of the growing oligosaccharide chain was removed using hydrazine/pyridine/acetic acid solution to furnish a nucleophile for the next glycosylation reaction. After four glycosylation reactions, the oligosaccharide was cleaved from the resin using Grubbs’ catalyst under an ethylene atmosphere to produce the final protected phytoalexin elicitor \( \beta \)-glucan in 89% yield over five steps.
9.05.7.2 Leishmania Cap Tetrasaccharide

Leishmania, which is transmitted to humans via the sand fly, affects millions of people worldwide. Automated solid-phase oligosaccharide synthesis was used to synthesize the tetrasaccharide cap of the antigenic glycosylphosphatidylinositol (GPI) found on the surface of the Leishmania parasite, providing the first example of the automated solid-phase synthesis of a branching glycan using functionalized monosaccharide building blocks with different LGs. Octenediol-functionalized Merrifield resin and three building blocks (4, 5, and 6, Scheme 8) containing C2 participating groups were employed in the synthesis of the tetrasaccharide cap. Mannosyl imidate 4 was glycosylated on to the linker using a catalytic amount of TMSOTf. Removal of the Lev group using hydrazine, followed by glycosylation with galactosyl phosphate 5 using a stoichiometric amount of TMSOTf gave the desired disaccharide. Hydrolysis of the C2 Ac under Zemplén conditions, followed by glycosylation with mannosyl imidate 6 gave the desired trisaccharide. A second hydrolysis and glycosylation with 6, followed by cleavage with Grubbs’ catalyst under an ethylene atmosphere gave the desired tetrasaccharide in 50% yield with approximately 85% coupling efficiency at each step.

9.05.7.3 Malaria Vaccine Tetrasaccharide

Malaria is caused by the parasite *Plasmodium*, which is transmitted by the mosquito. In 2010, approximately 216 million cases of malaria were recorded worldwide with more than 655,000 deaths reported (Data obtained from the World Health Organization). In 2002, a GPI toxin responsible for the pathogenesis of the parasite was identified and targeted for vaccine development. Shortly thereafter, the first automated solid-phase synthesis was reported. Four selectively functionalized imidate building blocks, 7, 1, 8, and 9, were required for this synthesis (Scheme 9). The use of acetates as temporary protecting groups and the need to install a second mannose residue at the C6 position of the reducing sugar required that imidate 7, with a nonparticipating group at C2, be employed for this synthesis. Glycosylation of mannosyl imidate 7 on to the octenediol-functionalized Merrifield resin using the ‘double coupling’ strategy and stoichiometric amount of TMSOTf led to a mixture of α and β anomers. This mixture was inconsequential since later steps in the synthesis of the vaccine candidate required hydrolysis of the pentenyl tether and refunctionalization of the reducing sugar. Methanolysis of the C6 Ac to the corresponding hydroxyl group using Zemplén conditions provided the corresponding nucleophile required for extension. Subsequent glycosylation/hydrolysis cycles with imidates 1, 8, and 9 gave the final tetrasaccharide in 44% yield after cleavage from the resin (approximately 82% coupling efficiency at each step). It is worth noting that the employment of building block 8 represented the first use of a silyl-protecting group in automated oligosaccharide synthesis.

9.05.7.4 Oligoglucosamines

β-(1→6) and β-(1→4) linked oligoglucosamines are expressed on the surface of pathogenic *Staphylococcus aureus* and are involved in a number of critical biological processes including cellular communication and adhesion. They are also antigenic. Therefore, interest in these compounds as potential vaccine candidates has prompted the development of rapid synthetic strategies to access them. The first automated solid-phase synthesis developed for the synthesis of a β-(1→6) glucosamine was...
Scheme 9  Malaria vaccine tetrasaccharide.
reported in 2002.\textsuperscript{41} An initial solution-phase screening led to the use of glucosylamine phosphate building block 10 functionalized with a participating phthalimido group (NPhth) at C2 and a temporary Lev group at C6. A 'double coupling' strategy was employed on the first-generation automated solid-phase oligosaccharide synthesizer. Glycosylation of the first building block with octenediol-functionalized Merrifield resin followed by removal of the Lev protecting group using a hydrazine/pyridine/acetic acid solution gave the free C6 hydroxy methyl group for extension. Two additional glycosylation/deprotection steps were required to access the final trisaccharide in 17% yield (55% coupling efficiency) after cleavage from the resin using Grubbs’ I catalyst in the presence of ethylene (Scheme 10). The lower yield is likely due to the steric hindrance and conformational constraints of the participating phthalimido group.

Development of the bifunctional linker and the second-generation automated solid-phase oligosaccharide synthesizer permitted the use of thioglycosides in the automated synthesis of β-(1→6) oligoglucosamines\textsuperscript{8} (Scheme 11). For this synthesis a 'double coupling' strategy was employed. Activation of glucosylamine thioglycoside 11 functionalized with a trichloroacetamide (TCA) participating group at the C2 amine and Fmoc group at C6 with a promoter system consisting of N-iodosuccinimide (NIS) and triflic acid (TfOH) in the presence of the bifunctional linker led to the initial monosaccharide bifunctional linker-functionalized resin. Hydrolysis of the Fmoc group using a solution of piperdine in DMF provided a free alcohol for extension. Subsequent glycosylation/deprotection cycles followed by base-mediated hydrolysis from the resin and reduction of the TCA groups led to the production of the hexasaccharide (five additional cycles) or the dodecasaccharide (11 additional cycles) in 58% yield with 92% coupling efficiency and 44% yield with close to 93% coupling efficiency, respectively. This synthesis indicated a marked improvement over the initial trisaccharide synthesis (Scheme 10). A similar strategy employing thioglycoside building block with a participating TCA group at C2 and an Fmoc group at C4 was used to synthesize a β-(1→4) hexasaccharide in 31% yield with 92% coupling efficiency and 44% yield with close to 93% coupling efficiency, respectively.
yield (not shown). The reduced yield for the \( \beta-(1 \rightarrow 4) \) hexasaccharide is presumably due to the increased steric encumbrance of the nucleophile.

**9.05.7.5 Core \( N \)-linked Glycan**

\( N \)-Linked glycans play critical roles in cell–cell recognition and communication in mammals. \( N \)-Linked glycans have also been implicated in tumor progression and metastasis, and the pathogenesis of viruses such as HIV,\textsuperscript{42} Ebola,\textsuperscript{43} and some coronaviruses.\textsuperscript{44} The pentasaccharide core of \( N \)-linked glycans, which consists of chitobiose (two \( \beta-(1 \rightarrow 4) \) linked glucosamine residues) followed by a \( C3/C6 \) branching \( \beta-(1 \rightarrow 4) \) linked mannose residue, presents a significant challenge due to the \( \beta-(1 \rightarrow 4) \)-mannosidic linkage to the chitobiose disaccharide.

The first automated solid-phase synthesis of the core \( N \)-linked glycan by Seeberger and co-workers in 2003 employed the first-generation automated solid-phase oligosaccharide synthesizer, octenediol-functionalized Merrifield resin and a ‘double coupling’ strategy using three selectively functionalized imidates (Scheme 12).\textsuperscript{45} The reducing glucosamine residue of the core chitobiose disaccharide was installed using imidate donor 12 functionalized with a participating TCA group at the C2 amine and a temporary Ac protecting group at C4 under standard conditions. Hydrolysis of the C4 Ac using Zemplé n conditions provided a nucleophile for subsequent glycosylation. In this synthesis, installation of the difficult \( \beta \)-mannosidic linkage was avoided by using disaccharide building block 13 with a participating TCA group at the C2 position and temporary acetate groups at the C3 and C6 positions. Hydrolysis of the acetates at C3 and C6 followed by double glycosylation with mannosyl imidate 1 gave the core pentasaccharide in 27% yield with approximately 73% coupling efficiency after cleavage from the resin using Grubbs’ catalyst.

![Scheme 12](image-url)  
**Scheme 12** Core \( N \)-linked glycan.

The development of the second-generation solid-phase oligosaccharide synthesizer, the bifunctional linker and advances in the synthesis of \( \beta \)-mannosidic linkages encouraged Seeberger and co-workers to revisit the synthesis of the \( N \)-linked glycan in 2012. Their new approach is illustrated in Scheme 13.\textsuperscript{8} The chitobiose core was installed using a ‘double coupling’ strategy with glucosylamine thioglycoside building block 14 functionalized with a TCA participating group at the C2 amine and a temporary Fmoc group on C4. Two glycosylation cycles were performed using an NIS/TfOH promoter system. Removal of the Fmoc group was accomplished between each cycle using piperidine to expose the next acceptor. The \( \beta \)-mannosidic linkage was then formed using mannosyl OCB building block 15 consisting of a nonparticipating benzylate at the C2 position, a temporary benzylidene acetal installed at the C4 and C6 positions, and a temporary silyl-protecting group at the C3 position, marking the first use of a
benzylidene acetal and an OCB donor in automation. Glycosylation of the chitobiose disaccharide with 15 using triflic anhydride (Tf₂O) and di-tert-butyl-4-methylpyridine (DTBMP), followed by removal of the silyl ether at C3 with TBAF and selective ring opening of the benzylidene acetal using diborane and dibutylboron triflate (Bu₂BOTf), gave the core N-linked trisaccharide which underwent subsequent glycosylation with mannosyl imidate 1. The final assembly of the core N-linked glycan was completed in 3.5% yield after cleavage from the resin under Zemplén conditions; the β-mannosidic linkage was installed with an α/β ratio of 1:3. Although the yields were far lower than the original synthesis of the core N-linked glycan, this work represents the first example of the direct generation of a beta-mannosidic linkage using an automated solid-phase platform.

9.05.7.6 Gb-3 and Globo-H

The cancer antigen glycosphingolipids Gb-3 (Scheme 14) and Globo-H (Scheme 15) are of interests for vaccine development. In addition, Gb-3 has recently been shown to play a role in HIV infectivity. Gb-3 and Globo-H were synthesized in record time using an automated solid-phase approach employing the first-generation automated oligosaccharide synthesizer. A cis α-galactosidic linkage linked to the axial C4 hydroxyl group of a second galactose residue is common motif to both glycans and presents a significant challenge in solution and in automation. After careful screening, it was determined that galactosyl imidate 18 (Gb-3) or galactosyl phosphate 19 (Globo-H), both containing a nonparticipating benzyl group at C2, could be used to selectively install this linkage efficiently. The protected oligosaccharides were subsequently assembled using octenediol-functionalized Merrifield resin under standard glycosylation conditions. Functionalized monosaccharides 16 through 18 were used to synthesize the Gb-3 trisaccharide (Scheme 14) in 46% yield with 77% coupling efficiency. Globo-H hexasaccharide was synthesized from building block 16, and building blocks 19 through 23 in 25 h (Scheme 15) and 30% yield with a coupling efficiency of 82% per step.

The development of the second-generation automated solid-phase oligosaccharide synthesizer and the bifunctional linker led to the first automated synthesis of iso Gb-3 (Scheme 16), a glycan of biological interest due to its homology with Gb-3. The synthesis of iso Gb-3 marked the first automated solid-phase oligosaccharide synthesis using a ‘double coupling’ strategy employing only differentially functionalized thioglycoside building blocks. The first glucose residue was installed by glycosylation of glucosyl thioglycoside building block 24, functionalized with a permanent participating C2 Piv group and a temporary Fmoc group at C4 with bifunctional linker-functionalized Merrifield resin using an NIS/TfOH promoter system. After hydrolysis of the Fmoc group using piperidine, subsequent glycosylation/deprotection cycles, first with galactosyl thioglycoside building block 25 with a participating pivaloate C2 protecting group and a temporary Fmoc group at C3, followed by glycosylation with galactosyl thioglycoside building block 26 with a nonparticipating C2 Bn group provided the desired glycan in 80% yield (93% yield...
Scheme 14  
Gb-3.
Scheme 15  Globo-H.
coupling efficiency) after cleavage from the resin using Zemplén conditions. The installation of the nonreducing terminal galactose building block 26 represented the greatest challenge.

![Scheme 16 Iso Gb-3.](image)

### 9.05.7.7 Blood Group Antigens

The type II Lewis blood group oligosaccharides Leι (hexasaccharide) and Leι–Lex (nonasaccharide) are of interest as potential cancer vaccines. Their complexity, including the number and different types of carbohydrate residues and challenging linkages, render them significant challenges for automated solid-phase oligosaccharide synthesis. The first automated synthesis of Leι and Leι–Lex employed the first-generation automated oligosaccharide synthesizer, an ester-linked octenediol-functionalized linker, and five differentially functionalized phosphate building blocks: 16, 27, 28, 23, and 29, under standard glycosylation conditions. Fmoc and Lev groups were used as temporary protecting groups whereas Piv and TCA groups were used as C2 directing groups with the exceptions of galactose building block 29, which used a C2 Fmoc group as both a temporary protecting and directing group, and fucose building block 23, which required a nonparticipating group to install the desired cis linkage. The synthesis of Leι is shown in Scheme 17. Successive 'double coupling' of building blocks 16, 27, 28, 23, 29, and 29 gave the desired hexasaccharide in 10% yield after cleavage from the resin using Zemplén conditions. After each double coupling, the Fmoc or Lev protecting group was removed using piperdine or hydrazine, respectively. The Leι–Lex nonasaccharide was synthesized using a similar protocol (Scheme 18) to produce the desired product in 6.5% yield after cleavage from the resin.

Sialic acid containing glycans mediate pathogen invasion and modulate important roles in cellular communication. Despite their significance, the chemical synthesis of these glycans has been limited by the challenging installation of the sialic acid residue, which is often accomplished in low yield and limited selectivity. The automated solid-phase oligosaccharide synthesis of Sialyl Lewisx (SLex), a tetrasaccharide involved in inflammation and metastasis, demonstrated that sialic acid residues could be installed in automation. The second-generation automated oligosaccharide synthesizer, bifunctional linker-functionalized Merrifield Resin and a 'double coupling' strategy employing three building blocks: glucosylamine thioglycoside 30 with a TCA participating group at C2 and temporary Fmoc and Lev protecting groups at C3 and C4; disaccharide imidate 31 containing an acetate participating group at C2; and fucosyl imidate 32 with a nonparticipating Bn group at C2 were required to complete the synthesis of SLex in just over 50% yield (80% coupling efficiency). The key to the success of this synthesis was the preparation of the disaccharide building block 31 incorporating the sialic acid residue. This method was applied to several sialyl lactose and sialyl lactosamine derivatives in excellent yields (Scheme 19).

### 9.05.7.8 Oligoglucone

An HPLC-based automated oligosaccharide synthesizer was recently employed by Demchenko and co-workers in the generation of several β-(1 → 6) linked oligosaccharides. The initial glucose residue containing a participating Bz group at C2 and a temporary trityl group at C6 was coupled to TentaGel resin using EDC and DMAP on column and then detritylated with wet TFA to expose
Scheme 17: Technology-Enabled Synthesis of Carbohydrates

1. 16, 2×5 equivalents TMSOTf CH₂Cl₂ −15 °C
2. Piperidine DMF

1. 27, 2×5 equivalents TMSOTf CH₂Cl₂ −15 °C
2. Piperidine DMF

1. 28, 2×5 equivalents TMSOTf CH₂Cl₂ −15 °C
2. Piperidine DMF

1. 23, 2×5 equivalents TMSOTf CH₂Cl₂ −15 °C
2. Piperidine DMF

1. 29, 2×5 equivalents TMSOTf CH₂Cl₂ −15 °C
2. Piperidine DMF

NaOCH₃

10% Le⁹
Scheme 18  Le<sup>α</sup>–Le<sup>α</sup>.
the C6 hydroxymethyl group for subsequent glycosylation (not shown). Four additional glycosylation/deprotection sequences using glucose building block 33, with a participating Bz at C2 and a temporary Fmoc group at C6 gave the desired pentasaccharide in 60% yield and a coupling efficiency of approximately 90% after cleavage from the resin using Zemplén conditions (Scheme 20). The fully deprotected pentasaccharide was reacetylated to simplify purification.

Scheme 19 Sialyl Leα.

Scheme 20 Oligoglucose.
9.05.7.9 Hyaluronan Oligosaccharides

Hyaluronic acids are glycosylaminoglycans composed of repeating units of glucuronic acid and N-acetylglycosamine monosaccharides. Hyaluronic acids function as molecular lubricants and have also been shown to play important roles in the inflammatory response, cell proliferation, recognition, migration, and adhesion. The first automated solid-phase synthesis of well-defined hyaluronic acids employed the second-generation automated synthesizer. Butenediol-functionalized Merrifield resin and a ‘triple coupling’ strategy using 3 equivalents of two di-tert-butylsilylidene-protected imidate building blocks 34 and 35 (Scheme 21) incorporating C2 participating groups (TCA for building block 34 and Bz for building block 35) and Lev groups as temporary protecting groups. Activation of glucosylamine imidate 34 using TfOH, which was demonstrated to be more efficient than TMSOTf, followed by hydrolysis of the Lev protecting group with hydrazine and iterative glycosylation reactions with disaccharide 35 gave the desired glycans in 18–26% yield (n = 3, 5, or 7) after cross-metathesis from the resin. Interestingly, the authors demonstrated that direct glycosylation of disaccharide 35 to the linker was unsuccessful, necessitating the synthesis of building block 34. In addition, removal of the glycan from the resin using cross-metathesis using Grubbs’ catalyst proved challenging due to the partial dechlorination of the TCA group. The use of a decoy substrate, trichloracetamide, was used to circumvent this unwanted reaction.

Scheme 21 Hyaluronic acid.

9.05.7.10 β-Mannuronic Acids Alginites

β-Mannuronic acid alginites are major components of the cell wall of algae. They are also major constituents of the exopolysaccharides of Pseudomonas aeruginosa, an opportunistic, nonsocomial gram-negative bacterium. The automated synthesis of tetra-, octa-, and dodeca-β-mannuronic acid alginites via automated solid-phase oligosaccharide synthesis was recently reported using the second-generation automated oligosaccharide synthesizer, butenediol-functionalized Merrifield resin, and a mannuronic imidate building block 36 functionalized with a nonparticipating Bn at C2 and a temporary Lev protecting group at C4 (Scheme 22). The major challenge with this synthesis was the selective installation of each β-mannosidic residue. TfOH was found to be the most effective promoter and the reaction was found to be completely selective at temperatures near the decomposition of the donor (−40 °C). Each glycosylation cycle required hydrolysis of the Lev group using hydrazine. The first oligosaccharide synthesized, a tetrasaccharide, was prepared using four glycosylation/deprotection cycles in 90% yield with approximately 98% coupling efficiency. The octasaccharide and dodecasaccharides were synthesized with 90% coupling efficiency at each step providing the final products in 55% and 40%, respectively.

9.05.7.10.1 Oligoarabinofuranosides

The first automated solid-phase oligosaccharide synthesis of oligoarabinofuranosides was recently reported. Oligoarabinofuranosides and their analogs are the major components of mycobacterial cell envelopes and play important roles in a number of
critical aspects of the life cycle of the mycobacterium.\textsuperscript{57–59} The versatility of the second-generation synthesizer combined with the use of the bifunctional linker provided for the synthesis of a series of linear and branched oligoarabinofuranosides from the sequential ‘double coupling’ of two thiofuranosides 37 and 38, both of which contained a Bz participating group at C2 and
temporary F-moc protecting groups at C5 (37) or C4 and C5 (38). The use of NIS/TFOH as a promoter system was shown to provide the best yields and selectivities. Piperidine was used to remove the Fmoc protecting groups in between coupling cycles. Overall, this process was shown to be efficient and rapid – the hexasaccharide in Scheme 23 was prepared with 63% yield and 92% coupling efficiency in 14 h.

9.05.8 Conclusion

Fully automated solid-phase synthetic oligosaccharide platforms and new linker strategies have streamlined access to a host of carbohydrate constructs including bacterial and viral antigens, cancer antigens, vaccine candidates, and N-linked oligosaccharides. This has led to an increase in the general understating of the fundamental roles of these oligosaccharides in a number of biological processes. Current research is directed at identifying monosaccharide building blocks that can be used to access the major glycoconjugate classes including N-linked, O-linked, glycolipids, and glycosylaminoglycans. These oligosaccharides will be used to study important carbohydrate–protein, carbohydrate–nucleic acid, and carbohydrate–carbohydrate interactions involved in normal and abnormal cellular processes. The development of rapid and efficient synthetic protocols for commercialization of these building blocks is the next critical step for expanding the automated solid-phase platform for oligosaccharide synthesis.

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