Top Curr Chem (2007) 278: 289–309
DOI 10.1007/128_2007_124

Published online: 11 April 2007

Automated Solid Phase Oligosaccharide Synthesis

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Abstract Of the three classes of biopolymers—nucleic acids, proteins and glycoconjugates, nucleic acids and proteins have seen the most breakthroughs in understanding their biological role, in part due to their ready availability. The automation of oligonucleotide and peptide synthesis has been fruitful in providing biologists and biochemists with pure, well-defined structures. This work reviews the recent developments in the automated synthesis of oligosaccharides, the third class of biopolymers. Both glycosyl phosphates and glycosyl trichloroacetimidates have been used successfully in the automated assembly of oligosaccharides employing an octenediol-functionalized polystyrene resin. The product was cleaved either by methanolysis of an ester bond or by olefin cross metathesis. Several biologically important carbohydrates have been synthesized by automation, in a fraction of the time needed to synthesize them by traditional methods. For example, the tumor associated antigens Lewis *Y*, Le^y-Le^x, were synthesized by automation. A *Leishmania* cap tetrasaccharide and a malaria toxin vaccine candidate were also assembled.

Keywords Automation · Carbohydrate synthesis · Oligosaccharides · Solid-phase

Abbreviations

CM	Cross-metathesis
DIPC	Diisopropylcarbodiimide
DMDO	Dimethyldioxirane
DMT	4,4′-Dimethoxytrityl
Fmoc	9-Fluorenylmethoxycarbonyl
Lev	Levulinoate
Phth	Phtalimido
Pip	Piperidine
TCA	Trichloroacetate
TMSOTf	Trimethylsilyl trifluoromethanesulfonate

1 Introduction

Three repeating biopolymers dominate the signal transduction processes in living organisms—nucleic acids, proteins and glycoconjugates. The role of nucleic acids and proteins has been the subject of intense research and development. The synthetic advances have fueled the emergence of several powerful tools for the identification of carbohydrate structure, function and interactions with other biomolecules. In turn, these tools, have allowed more discoveries. The pharmaceutical and biotechnology areas have seen several fundamental breakthroughs in genomics and proteomics. Detailed pictures of interactions of nucleic acids and proteins with each other or with themselves are starting to emerge (Fig. 1). These insights have led to the identification of many therapeutic approaches and opportunities. Glycomics, however, has been less understood, which leaves important areas of research and many therapeutic opportunities untapped.

Carbohydrates are mostly present in nature in the form of glycoconjugates (glycoproteins and glycolipids) [1]. Their role is unambiguously important but remains often vague. If the understanding of the biological role of carbohydrates is to approach that of nucleic acids and proteins, access to well-defined pure oligosaccharide structures will have to be improved. Compared to the other two biopolymers, the availability of a wide range of pure and defined oligosaccharides has been lagging behind. Several facts are responsible. First, there is no amplification system analogous to the polymerase chain reaction (PCR) of nucleic acids. Second, carbohydrates have been difficult to isolate from natural sources because of their often-heterogeneous nature. There is no recombinant DNA technique to produce them like for proteins. Third, the chemical synthesis of carbohydrates has been more challenging than that of peptides and oligonucleotides. Unlike amino acids and

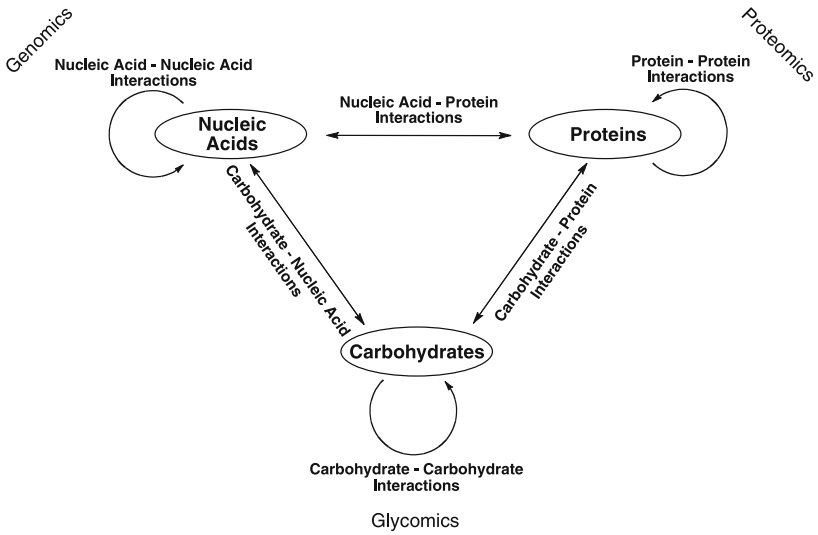


Fig. 1 Biopolymer interactions

nucleotides, monosaccharides have several possible points of attachment and the stereochemistry of the glycosidic bonds has to be defined. The lack of information regarding the structure of oligosaccharides has aggravated this situation. While the sequencing of samples of oligonucleotides and proteins is routine and has been automated, carbohydrate sequencing has been particularly challenging. More breakthroughs will be needed in this field even though progress is being made [2].

Both oligonucleotides [3] and peptides [4] can now be synthesized in an automated fashion, making them widely available. Non-experts can perform the synthesis of oligonucleotides and peptide on a routine basis. That development has allowed much progress in understanding the precise role of proteins and nucleic acids in biological systems.

Synthetic carbohydrate chemists have developed powerful methods to assemble complex carbohydrates [5]. Still, oligosaccharide synthesis remains technically challenging, and is limited to some expert labs. The number of different approaches that are available are not necessarily compatible with each other. A more unified and simplified approach for the synthesis of carbohydrates could tremendously benefit the field of glycomics by making a large variety of compounds available.

Automated oligosaccharide synthesis constitutes a useful tool. The basic idea of automation has now attracted the attention of several groups and drastically different approaches are being pursued in this direction. The group of Takahashi focused on automated parallel solution-phase synthesis of oligosaccharides based on a one-pot procedure [6]. A reactivity-based one-pot reaction has been pursued by Wong [7, 8]. Several groups are working on

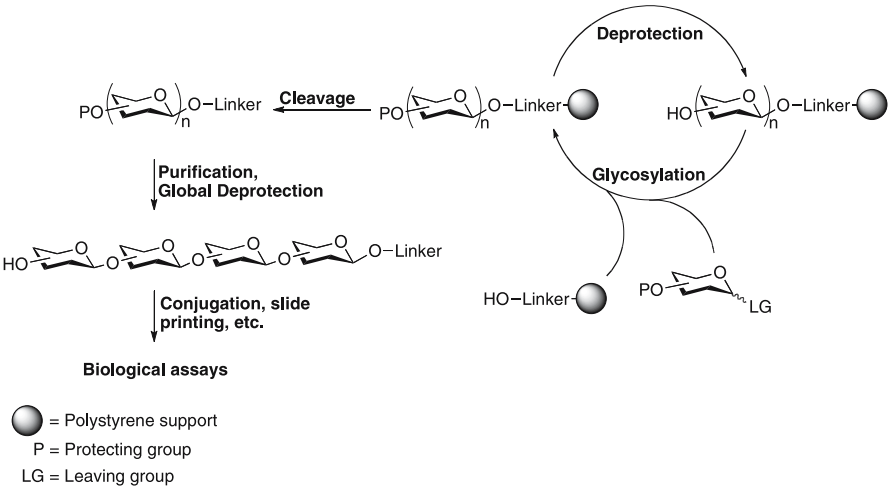


Fig. 2 Automated solid phase oligosaccharide synthesis

a fluorous linker-based strategy for carbohydrate synthesis that is potentially amenable to automation [9–11]. Nishimura's group developed the "Golgi" apparatus [12] based on enzymatic glycosylations. The basis of automation of oligonucleotides and peptides was the use of solid phase synthesis, where excess of reagents can be used to drive the coupling reactions to completion and no purification is necessary between the different steps. Solid phase oligosaccharide synthesis [13] has been performed since the early 1970s [14], but was not automated until 2001 [15]. The general scheme is depicted in Fig. 2. A linker is glycosylated followed by removal of excess reagent by washing. Then, a deprotection step reveals a new acceptor hydroxyl group. This cycle is repeated as many times as needed, after which, the resin-bound carbohydrate is cleaved from the solid support. Purification and deprotection yields an oligosaccharide that can be used in several different experiments. Several aspects of automated solid phase synthesis of oligosaccharides will be discussed here.

2
Automation Platform

Different instruments are now available for automated synthesis on solid phase. The Applied Biosystems peptide synthesizer ABI 433 (Fig. 3) was chosen as an initial platform due to its wide availability and the few modifications necessary for its application to oligosaccharide synthesis. The custom-made reaction vessel is constructed of jacketed glass to allow cooling. The building blocks are placed in cartridges to be delivered sequentially. The synthesizer allows for nine different solvents and reagents. A new, more versatile and less

Fig. 3 Modified peptide synthesizer

expensive platform, based on syringe pumps is currently being explored in our laboratory.

The programming of the instrument can be modified easily to accommodate different cycles of glycosylations, deprotections as well as different washing procedures.

3 Synthetic Strategy

Two general strategies are available for solid phase oligosaccharide synthesis, the acceptor-bound and the donor-bound strategy (Fig. 4). Both can also be combined in a bi-directional approach.

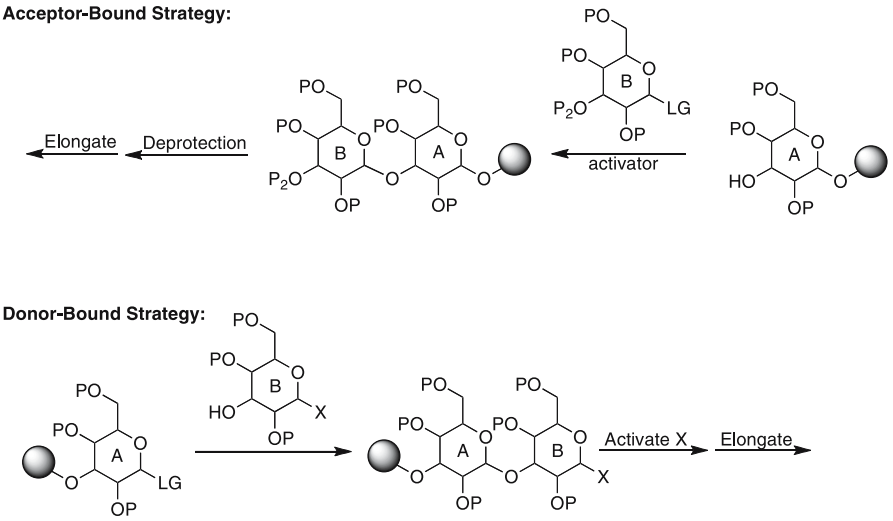


Fig. 4 Synthetic strategies

The acceptor-bound strategy has the "reducing end" of the oligosaccharide bound to the resin exhibiting a free alcohol. A building block and activator are added, commonly used in excess, to glycosylate the hydroxyl group. One or more hydroxyl groups on the newly incorporated sugar can be deprotected for elongation. The donor-bound strategy anchors the "non-reducing end" of the oligosaccharide to the solid support. An acceptor building block with a free alcohol and a stable anomeric leaving group precursor is added to the activated resin-bound donor. The newly added building block is then transformed into a donor and activated for elongation.

The acceptor-bound strategy permits more reactive building blocks, because decomposition of the donor results in soluble by-products that can be washed away from the resin-bound oligosaccharide. This strategy was ultimately used for automation.

4
Support and Linker

4.1
Solid Support

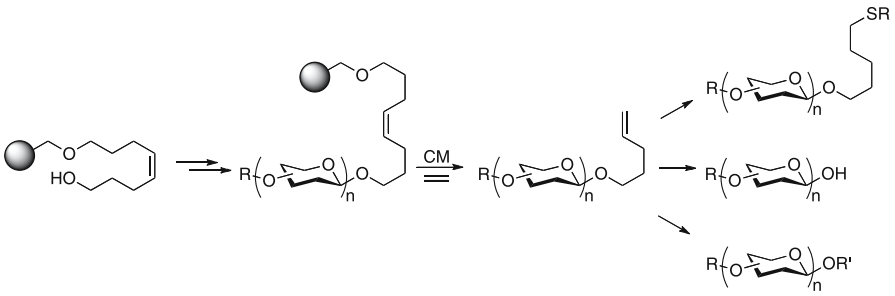
Non-soluble polymers were chosen as support for automation rather than soluble supports due to the ease of filtration through regular filters. A variety of polymer supports are commercially available including polystyrene-based

polymer, such as Merrifield [4] and TentaGel [16], and non-swelling controlled pore glass (CPG) support used in DNA synthesis.

Polystyrene resins have been successfully used in peptide synthesis and in organic solid-phase synthesis [17]. Polystyrenes exhibit good swelling properties in organic solvents that are commonly used in glycosylation reactions, such as dichloromethane and toluene. Swelling in more polar solvents is more difficult, but some polar solvents like DMF and THF exhibit good swelling properties. The overall stability of polystyrene towards acids and bases is an attractive feature for automated solid-phase carbohydrate synthesis.

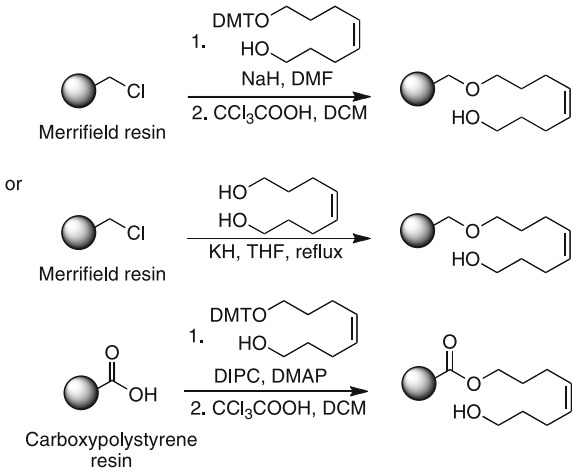
4.2 Linker

The polystyrene resin can be functionalized in various ways. The linker is of crucial importance for successful solid-phase synthesis because it has to remain intact during the synthesis cycles but should be easily cleaved in high yield with reagents that are not going to affect the synthesized carbohydrate. The two linkers used successfully for automated synthesis to date were both based on an octenediol linker (Scheme 1) attached to the resin via an ether [18] or an ester bond [19]. The octenediol linker has several advantages. First, the double bond is inert to many glycosylation strategies with the notable exception of thioglycosides. Second, the linker can be cleaved selectively by olefin cross-metathesis (CM). Finally, the resulting pentenyl group on the anomeric position can be activated [20] for glycosylation or hydrolysis or functionalized by radical-initiated addition of a thiol [21].



Scheme 1 Octenediol linker cleavage and modifications

The linker was synthesized from Merrifield resin or carboxypolystyrene and a DMT-protected diol (Scheme 2). The cleavage of the resulting DMT-protected linker allowed for UV quantitation of the resin loading [22]. The linker can also be installed directly from the diol [23]. The loading can be determined by Fmoc quantitation [24].



Scheme 2 Linker syntheses

5 Building Blocks

Carbohydrates contain a large variety of possible attachment and branching points. If one considers the ten mammalian monosaccharides [glucose (Glc), galactose (Gal), mannose (Man), sialic acid (Sia), glucosamine (GlcNAc), galactosamine (GalNAc), fucose (Fuc), xylose (Xyl), glucuronic acid (GlcA) and iduronic acid (IdoA)] 224 different building blocks would be

Table 1 The most abundant monosaccharide units found in mammalian glycans

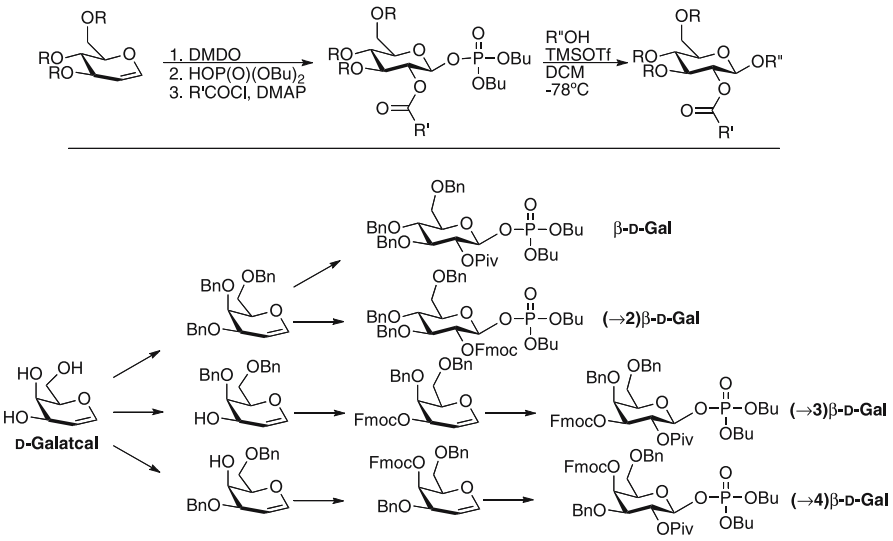
Monosaccharide unit	Abundance (%)
(→4)β-D-GlcNAc	21.7
(→3)β-D-Gal	9.5
α-D-Sia	8.8
α-L-Fuc	8.0
β-D-Gal	7.8
(→2)α-D-Man	7.1
(→3)(→6)β-D-Man	4.7
α-D-Man	2.8
β-D-GlcNAc	2.7
(→6)β-D-Gal	2.4
(→3)(→4)β-D-GlcNAc	2.3
(→2)(→4)α-D-Man	2.1
(→4)β-D-Glc	1.6
(→2)β-D-Gal	1.5

required theoretically to assemble any given oligosaccharide. A detailed statistical analysis (Seeberger, unpublished results) of the online database *glycosciences.de* [25] revealed that a far smaller number of building blocks would be necessary to construct a large fraction of the mammalian *N*- and *O*-linked oligosaccharides. In fact, a set of 25 building blocks would suffice to assemble 60% of the 3266 mammalian oligosaccharides contained in the database, and 65 building blocks would enable access to 90% of these oligosaccharides. A ranking of the most important monosaccharide units by abundance is depicted in Table 1.

Several building blocks giving access to these linkages have already been successfully used on solid support. A building block for automated solid phase synthesis needs to meet several criteria: it must contain a stable anomeric leaving group that can easily be activated. The temporary protecting group should easily be removed for the next glycosylation but needs to be stable to the glycosylation conditions. The glycosylation should be stereoselective, which usually implies an ester participating group on the C2 hydroxyl. The two types of anomeric leaving groups used for automated synthesis are glycosyl phosphates and glycosyl trichloroacetimidates.

5.1 Glycosyl Phosphate Building Blocks

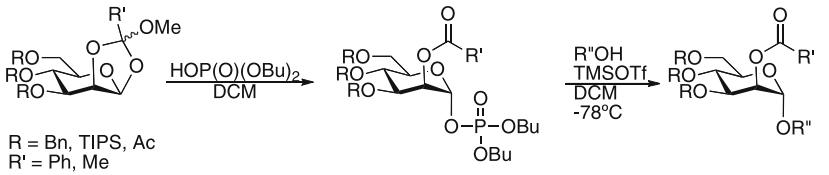
Glycosyl phosphates are efficient glycosylating agents for oligosaccharide synthesis [26–32] and can be readily obtained from glycals [33] via a one-pot



Scheme 3 One-pot formation of different glycosol phosphates

procedure [30, 31] (Scheme 3). The glycal precursors simplify the protection scheme as only three hydroxyls have to be differentiated. For example, galactal can yield four different building blocks - terminal, 2-linked, 3-linked, or 4 linked in only two or three steps.

The glycosyl phosphates can also be obtained from orthoesters in a single, high-yielding reaction (Scheme 4) [34].

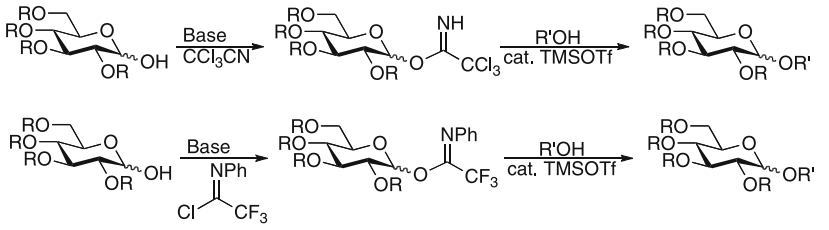


Scheme 4 Glycosyl phosphate synthesis from orthoesters

The activation of a phosphate group requires a stoichiometric amount of Lewis acid. Glycosyl phosphates can generally be stored for several months in the freezer without significant degradation.

5.2 Glycosyl Trichloroacetimidate Building Blocks

Glycosyl trichloroacetimidates [35] have been used most extensively in solution phase chemistry. These building blocks are easily obtained from the free anomeric lactol and can be activated under mild conditions with a catalytic amount of Lewis acid (Scheme 5). More recently, a variation of this important class of glycosylating agent gained some attention. The *N*-phenyl trifluoroacetimidate is as easily prepared and is activated under similar conditions [36]. This type of anomeric leaving group has shown advantageous reactivities when compared to the trichloroacetimidate [37]. This glycosylating agent can also be used on solid support [23].

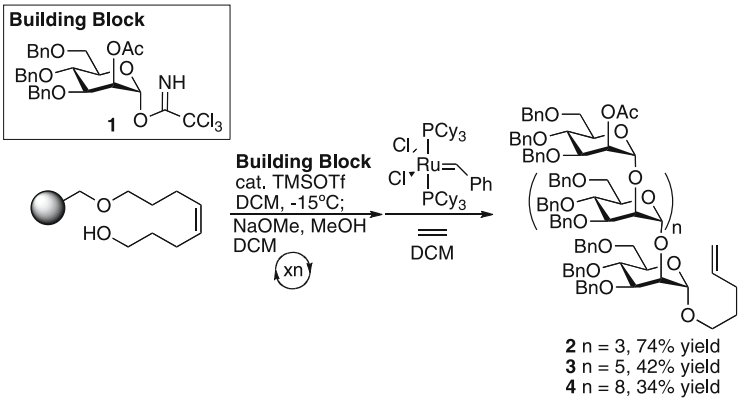


Scheme 5 Glycosyl trichloroacetimidate and *N*-phenyl trifluoroacetimidate building blocks

6
Automated Synthesis

6.1
α-Mannan and β-Glucan

The Seeberger group reported the first automated synthesis of oligosaccharides in 2001 [15] using the synthesis of poly α-(1→2) mannosides (Scheme 6) and a phytoalexin elicitor β-glucan (Scheme 7) as examples. The polymannoside was assembled using mannosyl trichloroacetimidate building block **1**. The building block was used in a ten-fold excess to react with the octenediol functionalized resin in the presence of a catalytic amount of trimethylsilyl trifluoromethanesulfonate for 30 minutes. The glycosylation was repeated once and the resin was washed with several solvents. The C2 hydroxyl was deprotected by treatment of the resin with an excess of sodium methoxide in a methanol/dichloromethane mixture. This sequence was repeated a number of times to obtain penta-, hepta- and deca-saccharide **2**, **3** and **4** in good overall yield after cross-metathesis with ethylene.



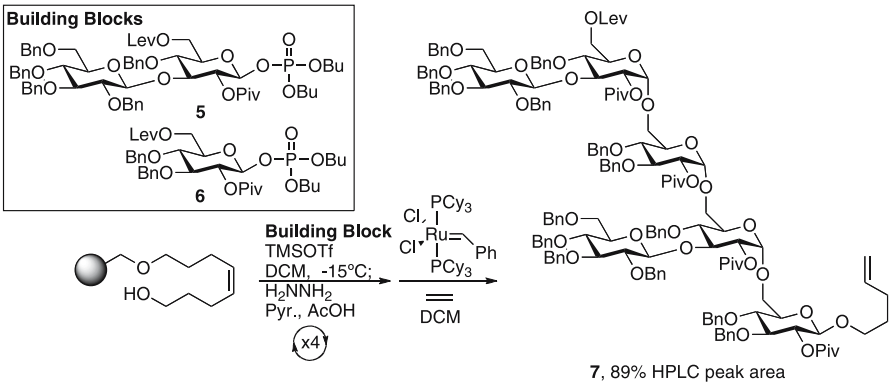
Scheme 6 Automated polymannose synthesis

The coupling cycle used for this synthesis is shown in Table 2. One cycle is completed in 2.6 h. The cycle is repeated for each building block incorporation such that the pentasaccharide was assembled in only 14 h.

Soybean plants can produce the antibiotic phytoalexin when exposed to a fungal β-glucan. A phytoalexin elicitor hexasaccharide was synthesized by automation using glycosyl phosphate building blocks (Scheme 7). The functionalized resin was submitted to building blocks in a five-fold excess with stoichiometric amounts of trimethylsilyl trifluoromethanesulfonate for 15 minutes at – 15 °C. The resin was washed and treated with hydrazine to affect the deprotection of the C6 hydroxyl. The sequence was repeated three times,

Table 2 Coupling cycle for automated synthesis using trichloroacetimidate building blocks and acetate protecting group

Step	Function	Reagent	Time (min.)
1	Couple	10 eq. Building block and 0.5 eq. TMSOTf	30
2	Wash	CH_2Cl_2	6
3	Couple	10 eq. Building block and 0.5 eq. TMSOTf	30
4	Wash	CH_2Cl_2	6
5	Wash	1:9 Methanol:CH_2Cl_2	6
6	Deprotection	2 x 10 eq. NaOMe (1:9 Methanol:CH_2Cl_2)	60
7	Wash	1:9 Methanol:CH_2Cl_2	4
8	Wash	0.2M Acetic acid in THF	4
9	Wash	THF	4
10	Wash	CH_2Cl_2	6

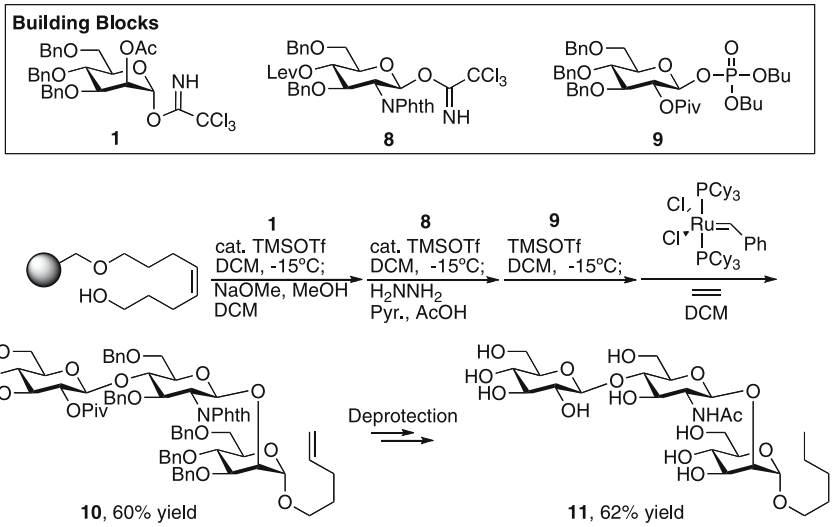


Scheme 7 Automated phytoalexin elicitor hexasaccharide synthesis

alternating the building blocks. A crude HPLC after cross-metathesis revealed a product peak accounting for 89% of the mixture.

These syntheses established the automated synthesis as an efficient and rapid way of assembling oligosaccharides. Both trichloroacetimidates and phosphates were competent donors and acetate and levulinoate could be used as temporary protecting groups for the synthesis. These two approaches were combined for an automated synthesis of a trisaccharide on a 100 μmol scale (Scheme 8).

Product **10**, obtained in 60% yield, was then fully deprotected to afford trisaccharide **11** in 62% yield or 37% overall yield from the functionalized resin. This example demonstrates the validity of the automated solid phase method to produce oligosaccharides in their deprotected form, ready for biological assays.



Scheme 8 Automated synthesis of a deprotected trisaccharide

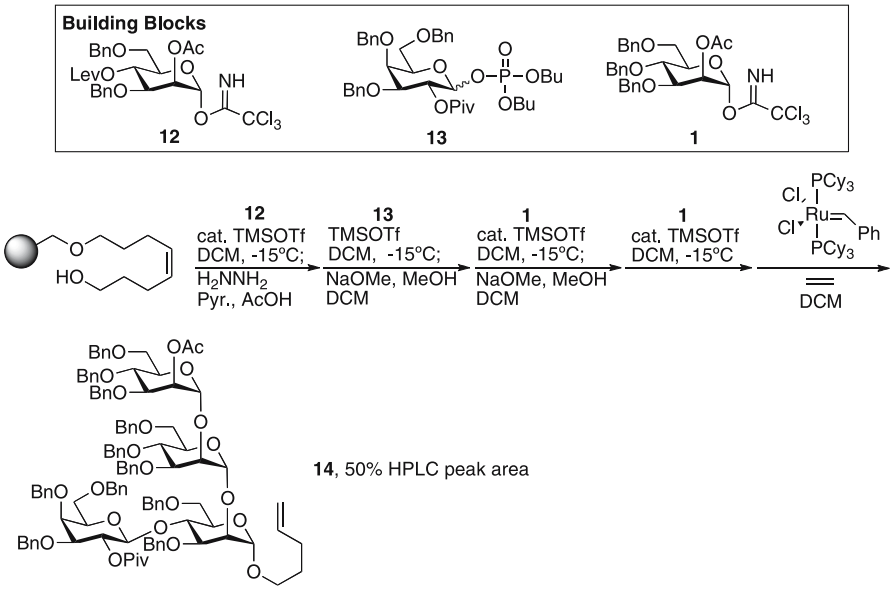
6.2
Leishmania Cap Tetrasaccharide

Leishmaniasis is a disease that affects over 12 million people worldwide. One of the carbohydrates that is exposed on the *Leishmania* parasite surface is a structure consisting of a glycosylphosphatidylinositol (GPI) anchor, a repeating phosphorylated disaccharide and tetrasaccharide cap **14**. The tetrasaccharide cap was synthesized by automation [38] using three building blocks (Scheme 9).

Levulinoate and acetate groups were used as temporary protecting groups during this assembly. Branching was achieved by having both ester protecting groups on the same building block and deprotecting the levulinoate in the presence of the acetate using hydrazine. The tetrasaccharide was the major product in the mixture as determined by HPLC. This synthesis demonstrates the possibility of producing branched structures by automation, with the use of orthogonal ester protecting groups.

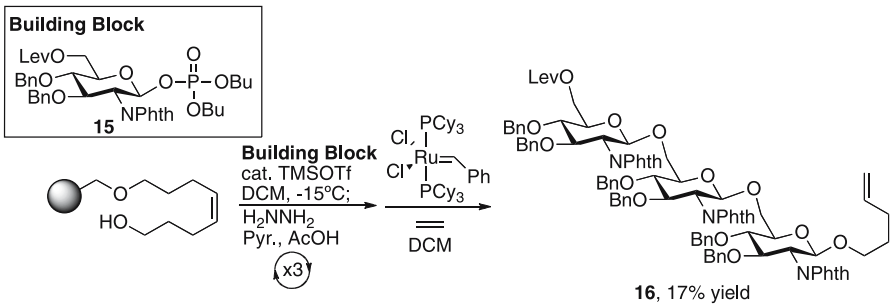
6.3
Oligoglucosamine

Glucosamines are involved in several important biological processes including cell-cell adhesion and immune response. Poly $\beta(1\rightarrow6)$glucosamines in particular, is an in vivo-expressed surface polysaccharide in human *Staphylococcus aureus* infections [39] and is a vaccine candidate [40]. A methodology



Scheme 9 Automated *Leishmania* cap tetrasaccharide synthesis

to assemble polyglucosamine by automation was developed [32]. A solution phase study was first conducted to identify a suitable building block for this purpose. A participating phtalimido group as a masked nitrogen and a phosphate as the leaving group in building block **15** were identified as optimal (Scheme 10).

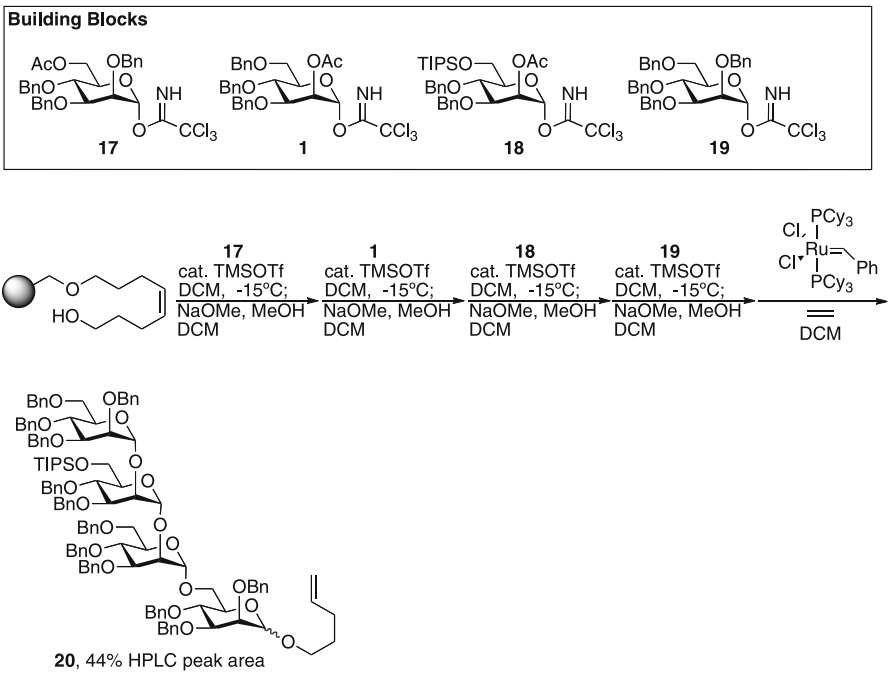


Scheme 10 Automated triglucosamine synthesis

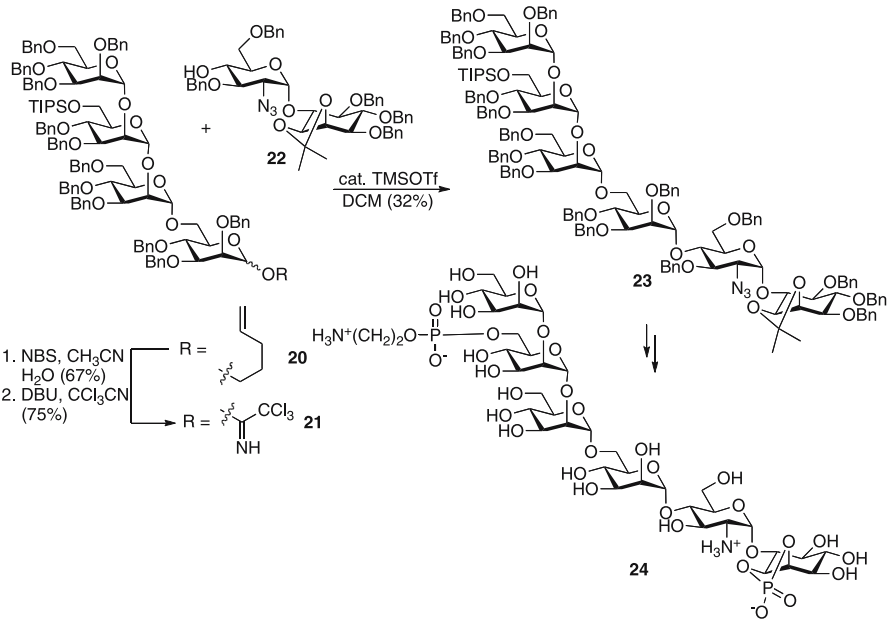
The synthesis was performed by treating the resin with five equivalents of building block and five equivalents of trimethylsilyl trifluoromethanesulfonate as activator. Deprotection of the levulinoate between glycosylations was performed with hydrazine. Cross-metathesis afforded the trisaccharide in 17% overall yield, along with 9% of the disaccharide deletion sequence.

6.4
Malaria Vaccine Tetrasaccharide

A GPI toxin [41] is responsible for the pathogenesis of malaria that induces cytokine and adhesin expression in macrophages and the vascular endothelium. The glycan portion of this GPI was synthesized in solution and conjugated to a protein to form a vaccine construct. The vaccine candidate protected mice against malaria pathogenesis. An automated synthesis of this carbohydrate and analogues would allow for rapid screening of various vaccine candidates. To this end, the synthesis of the tetramannose portion of the glycan was automated (Scheme 11) [42]. The building blocks were chosen considering the overall protecting group strategy of the complete malaria vaccine glycan. Benzyl and triisopropylsilyl ethers were used as orthogonal permanent protecting groups, whereas acetyl groups were used as the temporary protecting group to be removed prior to elongation. Four building blocks were needed. The glycosylation of building block **1** with the octenediol resin was not selective, as no participating group was used. However, further functionalization of the anomeric position renders this selectivity inconsequential. The automated synthesis afforded the product as the major



Scheme 11 Automated tetramannose synthesis



Scheme 12 Elaboration to the unprotected vaccine candidate **24**

component of the cleaved mixture. The mixture of anomers was purified by HPLC and further elaborated in solution (Scheme 12).

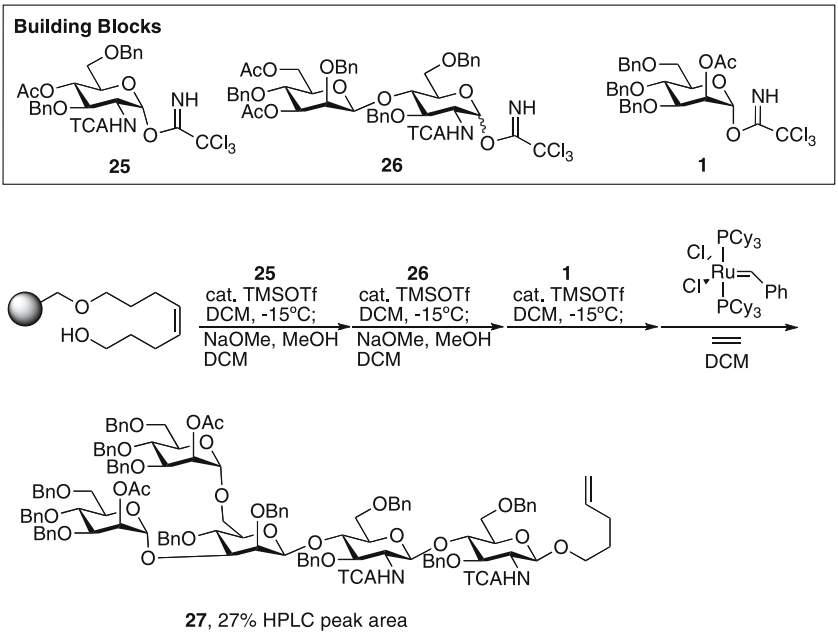
Although direct couplings of pentenyl glycosides are reported [20], a model donor failed to glycosylate the pseudo disaccharide **22.** The pentenyl glycoside **20** was converted to trichloroacetimidate donor **21** that was then used to glycosylate the disaccharide in moderate yield. Hexasaccharide **23** was further functionalized and deprotected to afford the antigenic glycan portion of the malaria toxin (compound **24**).

This synthesis opens the possibility of assembling various constructs for evaluation as vaccines. The anomeric pentenyl was successfully converted to a trichloroacetimidate donor, opening the way to building large carbohydrates from smaller fragments.

6.5 *N*-Linked Glycoprotein Core Pentasaccharide

There are three classes of asparagine-linked, or *N*-linked glycans: high-mannose, hybrid and complex-type mannans. All possess an identical core pentasaccharide **27**, comprised of a chitobiose ($\beta(1\rightarrow4)$diglucosamine) followed by a β-mannose branching two α-mannose residues. These glycans are not only found in mammals, but also on the glycoproteins of pathogens, including HIV [43], Ebola [44] and some coronaviruses [45].

The core pentasaccharide has been the subject of several solid-phase syntheses [46, 47] prior to our automated synthesis (Scheme 13) [48]. The automated assembly was performed using three different glycosyl trichloroacetimidate building blocks. The challenging β-mannosyl linkage was installed in solution and the disaccharide was transformed into building block **26.** Acetyl groups were used as temporary protecting groups. The resin was glycosylated twice with 3.5 equivalents of building block except for building block **26,** where the glycosylation was repeated three times. After cross-metathesis, the product peak area was 27% and could be isolated by preparative HPLC.

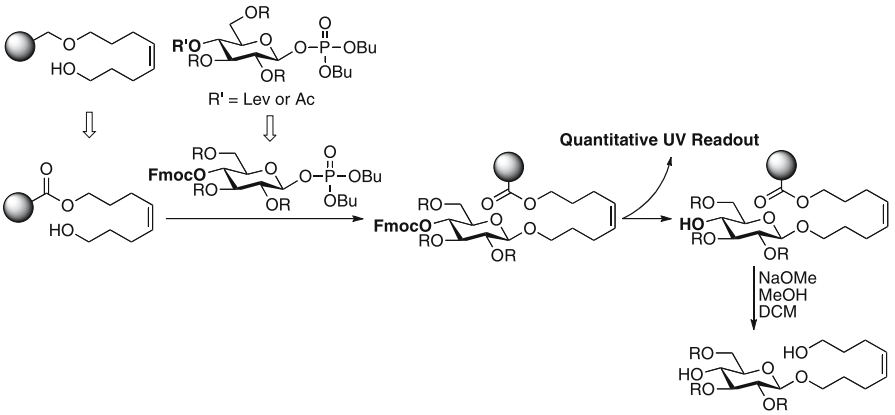


Scheme 13 Automated core pentasaccharide synthesis

6.6 Type II Lewis Blood Group Oligosaccharides

Lewis^y hexasaccharide and Le^y-Le^x nonasaccharide are tumor markers that are currently being evaluated as cancer vaccines [49]. Their complex structures constitute a challenge for synthesis both in solution and on solid phase [50, 51]. The successful automation [19] of this synthesis came after a solution phase [52] study and some modification to the previous automated syntheses (Scheme 14).

First, the resin was modified to include an ester functionality between the linker and the resin. This allowed for faster and more reliable cleavage

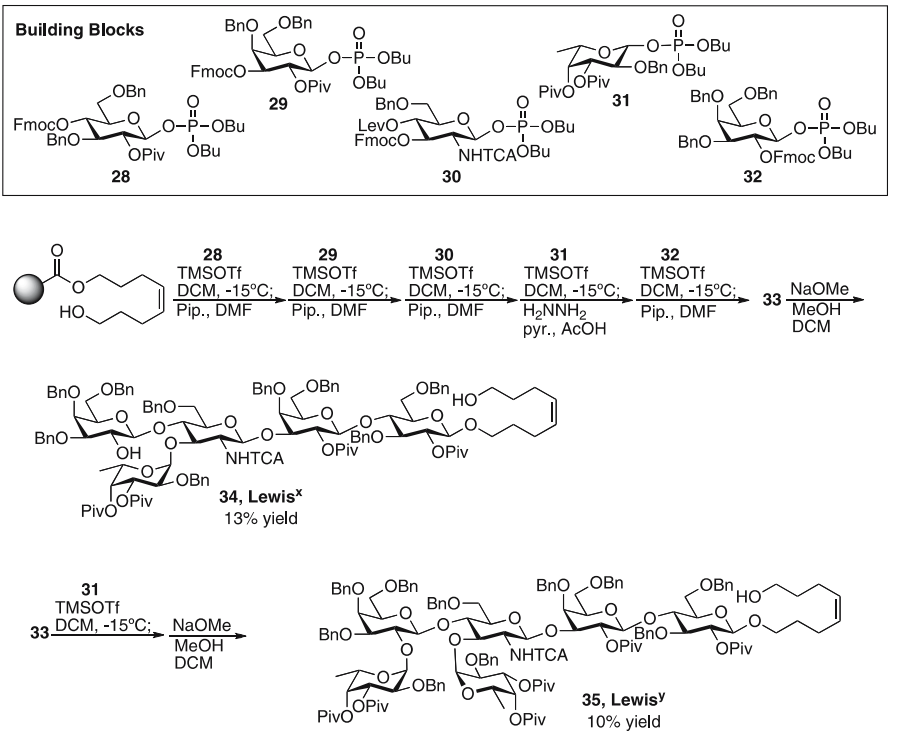


Scheme 14 Modifications from previous syntheses

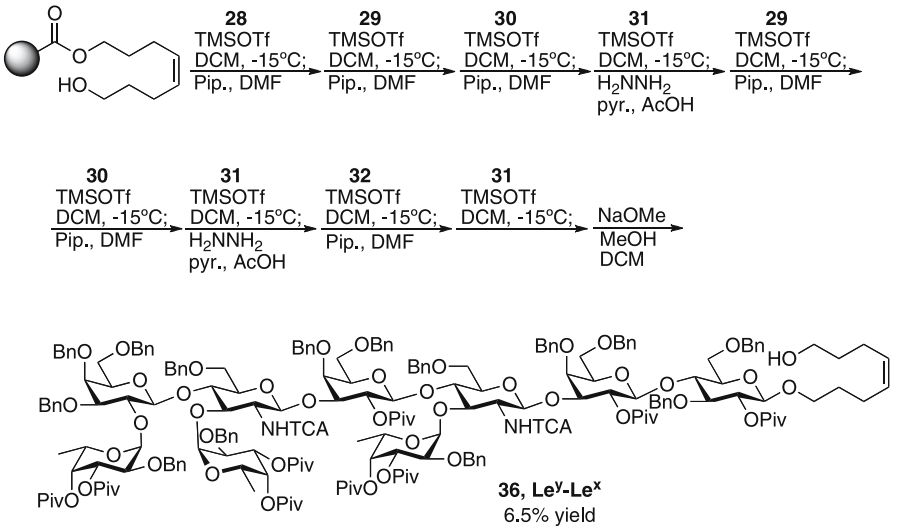
of the carbohydrate after the synthesis. The presence of this ester precluded the use of acetate or benzoate as a temporary protecting group since the conditions to remove them would also cleave the oligosaccharide from the support. Two orthogonal protecting groups had to be used for the branching glucosamine building block. The Fmoc group [46, 51, 53] can be removed by treatment with weak amine bases such as piperidine or triethylamine, and is orthogonal to levulinoate esters that require stronger basic conditions. The Fmoc deprotection is very rapid and can be quantitated by UV analysis. This procedure has the advantage of providing immediate feed-back regarding the efficiency of each glycosylation, information that is particularly important at the development stage of the synthesis.

The synthesis of Lewis *X* pentasaccharide **34** and Lewis *Y* hexasaccharide **35** required five different building blocks (Scheme 15). The glucose and galactose building blocks **28**, **29** and **32** were assembled efficiently using the previously described one-pot method based on the use of glycals. The glycosylations were performed with the standard five equivalents at – 15 °C repeated once, except for the glucosamine building block **30**, that was incorporated using three times 3.5 equivalents. This protocol was devised to ensure complete glycosylation despite competing decomposition of the building block under glycosylation conditions. The resin-bound pentasaccharide **33** could be released from the resin to afford pentasaccharide **34** in 13% overall isolated yield or fucosylated with building block **31** to give hexasaccharide **35** in 10% overall isolated yield.

The $Le^{y}Le^{x}$ dimer **36** was assembled in a similar fashion (Scheme 16) to give the nonasaccharide in 6.5% overall isolated yield after nine glycosylations, eight deprotection steps and one cleavage and purification step. The size and complexity of this oligosaccharide underscores the power of this methodology.



Scheme 15 Automated Lewis^x and Lewis^y synthesis



Scheme 16 Automated Le^y-Le^x synthesis

7 Conclusion

Automated solid phase synthesis has provided access to a broad range of biologically relevant oligosaccharides including cancer antigens, vaccine candidates and the *N*-linked core pentasaccharide, in a fraction of the time it would take to produce them by conventional means. Rapid access to a vast number of oligosaccharides has become possible and is a powerful tool for glycomics research that is currently being further improved.

Acknowledgements Funding from the ETH Zürich, the Swiss National Science Foundation (SNF Grant 200121-101593) are gratefully acknowledged.

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