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The Logic of Automated Glycan Assembly

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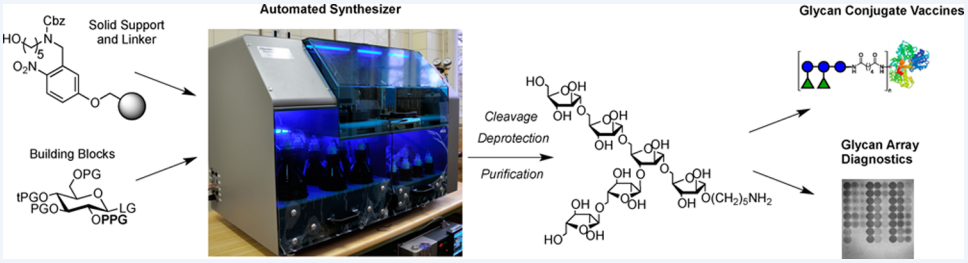

CONSPECTUS: Carbohydrates are the most abundant biopolymers on earth and part of every living creature. Glycans are essential as materials for nutrition and for information transfer in biological processes. To date, in few cases a detailed correlation between glycan structure and glycan function has been established. A molecular understanding of glycan function will require pure glycans for biological, immunological, and structural studies. Given the immense structural complexity of glycans found in living organisms and the lack of amplification methods or expression systems, chemical synthesis is the only means to access usable quantities of pure glycan molecules. While the solid-phase synthesis of DNA and peptides has become routine for decades, access to glycans has been technically difficult, time-consuming and confined to a few expert laboratories.

In this Account, the development of a comprehensive approach to the automated synthesis of all classes of mammalian glycans, including glycosaminoglycans and glycosylphosphatidyl inositol (GPI) anchors, as well as bacterial and plant carbohydrates is described. A conceptual advance concerning the logic of glycan assembly was required in order to enable automated execution of the synthetic process. Based on the central glycosidic bond forming reaction, a general concept for the protecting groups and leaving groups has been developed. Building blocks that can be procured on large scale, are stable for prolonged periods of time, but upon activation result in high yields and selectivities were identified. A coupling–capping and deprotection cycle was invented that can be executed by an automated synthesis instrument. Straightforward postsynthetic protocols for cleavage from the solid support as well as purification of conjugation-ready oligosaccharides have been established. Introduction of methods to install selectively a wide variety of glycosidic linkages has enabled the rapid assembly of linear and branched oligo- and polysaccharides as large as 30-mers. Fast, reliable access to defined glycans that are ready for conjugation has given rise to glycan arrays, glycan probes, and synthetic glycoconjugate vaccines. While an ever increasing variety of glycans are accessible by automated synthesis, further methodological advances in carbohydrate chemistry are needed to make all possible glycans found in nature. These tools begin to fundamentally impact the medical but also materials aspects of the glycosciences.

1. INTRODUCTION

Carbohydrates are the predominant component of biomass on earth since they are an integral part of all living things, including animals, plants, and bacteria.[1] Carbohydrates are important for many structural and functional aspects of biology but remain relatively poorly understood. The glycosciences remain largely descriptive. In stark contrast, the other two classes of repeating biopolymers, oligonucleotides (DNA and RNA) and proteins, are extremely well understood at the molecular level. Several factors complicate the study of glycans. Carbohydrates are structurally very diverse since they are found typically as part of glycoconjugates where a glycan and a protein (= glycoprotein), a glycan and a lipid (= glycolipid), or a glycan, a lipid, and a protein (= glycosylphosphatidylinositol (GPI) anchor) are combined. In addition, glycans are not just linear polymers such as oligonucleotides or proteins, but rather they are branched (Figure 1). Each glycosidic linkage that connects two monosaccharides is a new stereogenic center in contrast to phosphate diesters in oligonucleotides or amide linkages in proteins. The three-dimensional structure of glycans can be very diverse when one considers the combination of stereoisomers and branching. To further complicate matters for glycoscientists, hundreds of monosaccharides are known to serve as building blocks compared with fewer amino acids or nucleotides that are used to construct the other biopolymers.

The first half of the 20th century saw immense successes in the discovery and study of glycans. With the advent of molecular biology in the 1970s where tools to manipulate DNA and thereby also proteins became available, many biologists

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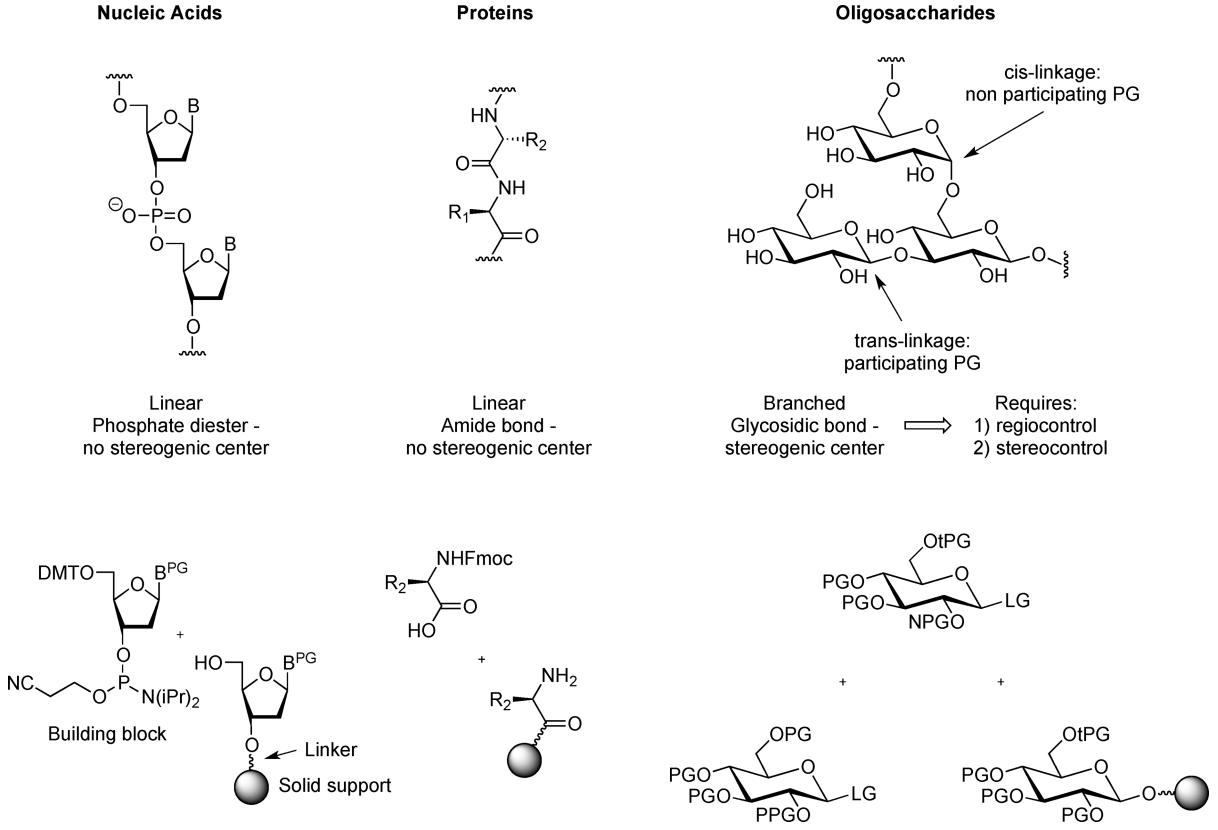

Figure 1. Structure of nucleic acids, peptides, and oligosaccharides guides automated solid phase synthesis. Abbreviations: PG, protecting group; LG, leaving group; TPG, temporary protecting group; NPG, nonparticipating protecting group; PPG, participating protecting group.

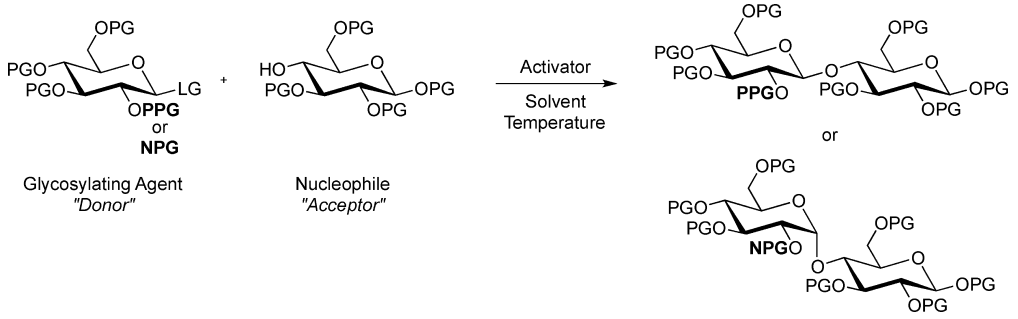

Figure 2. Glycosylation reaction as central transformation of glycan assembly. The stereochemical outcome of the reaction depends on the protecting groups, the anomeric leaving group, activator, temperature, and solvent.

shifted their focus to problems involving oligonucleotides and proteins. Automated sequencing and automated synthesis have been key to progress in proteomics and genomics.

Following the logic of the physicist Robert Feynman "I can only understand what I can create", automated access to peptides and oligonucleotides has changed the way the biology of these biopolymers is approached. The synthesis of a long peptide[2] or oligonucleotide[3] was a heroic accomplishment in the 1960s. The methods for solid-phase synthesis developed by Merrifield in the early 1960s for peptides[4] and by Caruthers in 1980 for DNA,[5] together with the advent of high-performance liquid chromatography (HPLC), provided access to defined oligomers. Rapid access to oligonucleotides gave rise to other important techniques such as the polymerase chain reaction (PCR) amplification method.[6] In addition to native structures, peptide and oligonucleotide analogs are useful tools.

The lack of pure glycans has greatly hampered the assignment of structure−function relationships in the glycosciences. Neither bacterial expression systems, such as exist for proteins, nor amplification methods analogous to PCR for oligonucleotides exist. Access to pure glycans has been extremely difficult. Due to the immense diversity of glycans on the cell surface, purification of pure molecules is at best very challenging and often impossible. Consequently, synthetic access to glycans is frequently the only way to procure the molecules to be studied.

The molecular complexity and immense diversity of glycans has posed a tremendous challenge to organic chemists. Heroic total synthesis efforts were required to create a class of molecules that from a retrosynthetic perspective generally is considered "boring" and "simply" required the installation of glycosidic linkages and protecting group manipulations.[7] For the fundamental glycosciences and their applications in

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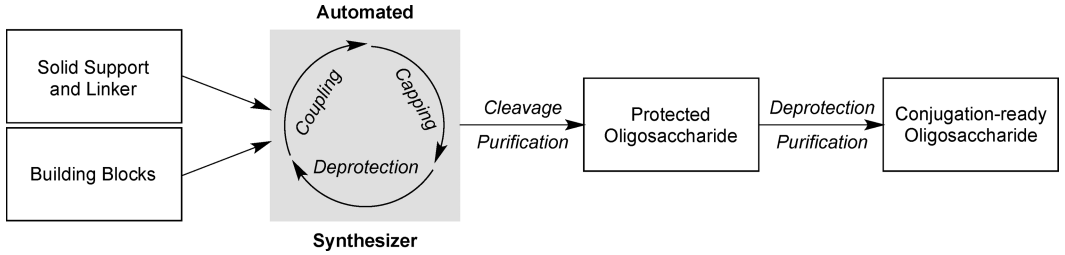

Figure 3. Schematic overview of the automated glycan assembly process.

medicine and material science to advance, the procurement of milligram quantities of glycans has to become rapid and reliable. Molecular tools and novel, non-natural structures will help us to establish structure−function relationships and to expand on what nature provided.

Methods for the chemical synthesis of oligosaccharides and glycoconjugates improved to the point where glycans representative of the major classes of carbohydrates are accessible, even though they often require months or years to complete.[8] The advent of reliable automated glycan assembly summarized here establishes synthetic chemistry as the enabling technology for the glycosciences.

2. STRATEGIC CONSIDERATIONS

From a retrosynthetic point of view, complex glycans are simple to analyze with the glycosidic linkage as the only type of disconnection. Glycosidic bond formation is key to the synthesis of all classes of glycans (Figure 2).[9] The glycosylation reaction involves a nucleophile ("glycosyl acceptor") that attacks the anomeric center of the glycosylating agent ("glycosyl donor") after the leaving group in the anomeric position of the donor is activated. All hydroxyl and amine groups are masked by protecting groups to ensure that only the desired, unprotected hydroxyl group(s) is (are) glycosylated. The size, electronic properties, and conformational nature of the protecting groups as well as the solvent, the temperature, and the activator have tremendous impact on the reactivity and the stereochemical outcome of the glycosylation reactions. Attack of the anomeric carbon occurs after activation either on the oxonium ion intermediate or via a direct S_N2-type displacement. Over the course of 110 years, a host of anomeric leaving groups have been developed.[10] In the past three decades, glycosyl imidates,[11] thioglycosides,[12] or glycosyl phosphates[13] that are activated by Lewis acids have replaced glycosyl halides that are activated by metal salts.

The strategic simplicity but operational complexity and unpredictability of glycosylation reactions rendered carbohydrate chemistry a specialized area of organic synthesis that was considered unattractive by many. The influence of the protective groups on the reactivity of glycosylating agents and nucleophiles offered opportunities. Glycosylating agents that carry mainly electron donating protecting groups such as benzyl ethers ("armed") are more reactive than those that are predominantly protected with electron withdrawing benzyl ester groups ("disarmed").[14] Knowledge of the relative reactivities of many glycosylating agents was the basis for the sequential "one-pot" combination of building blocks that can serve as glycosylating agents as well as nucleophiles.[15] One-pot glycosylations have accelerated the assembly of glycans up to hexasaccharides.[16] Generally, reactivity differences of glycosylating agents complicate glycan syntheses because different building blocks require adjustments in reaction temperature and time. Glycan syntheses would become more predictable if all building blocks would react at similar temperatures and times.

Control over the stereochemical outcome of glycosidic bond formation is quite reliable for *trans*-glycosidic linkages where placement of a participating protective group at the C-2 hydroxyl or amine blocks one face of the oxonium ion, while the nucleophile can attack only from one side. Typically, esters or amides serve as participating protecting groups (PPG). The presence of participating protecting groups in any other position of a glyosylating agent can potentially influence the stereochemical outcome of glycosylations.[17] The formation of *cis*-glycosidic linkages cannot benefit from the use of participating protecting groups (PPG) but requires the use of nonparticipating groups (NPGs) such as benzyl ethers or azides in the C-2 position. Without the influence of participating groups, the thermodynamically more stable product, typically the α-glycoside, is formed due to the anomeric effect. *cis*-β-Glycosidic linkages, such as β-mannosides, are the most challenging to construct because chemists cannot use participating protecting groups and have to fight the anomeric effect. Construction of these challenging linkages depends heavily on the conformation of the glycosylating agent, and conformational locking has proven helpful.[18] All strategic considerations related to glycan assembly have to focus on the glycosylation reaction as the central challenge.

The process of automated glycan assembly (Figure 3) relies on a solid support equipped with a linker that is used to successively install one building block after another using an automated synthesizer. Solid-phase syntheses benefit from mass action as reactive building blocks are used in excess to drive the reactions to completion while unreacted reagents can be simply washed away, and purification is performed only at the stage of the final product. On the other hand, solid-phase syntheses mandate very high coupling yields and superb stereoselectivity since purification is not possible after each step and every loss in coupling yield is carried through to the end of the synthesis. Either the glycosylating agent ("donor-bound approach")[19] or the nucleophile ("acceptor-bound approach")[20] can be attached to the solid support. The latter approach is now the method of choice, because the reactive glycosylating agent that may undergo nonproductive side reactions can be used in excess to ensure higher yields. For oligonucleotide and peptide syntheses, also the reactive agent is added to the growing support-bound polymer chain that serves as nucleophile (Figure1). The first monosaccharide building block is connected to the solid support via a linker that should be viewed as a protective group that is attached to the support matrix.[21] The choice of the linker is crucial since it determines the conditions that can be utilized throughout the entire synthesis without cleaving it but it has to be efficiently cleaved at the end of the synthesis. The temporary

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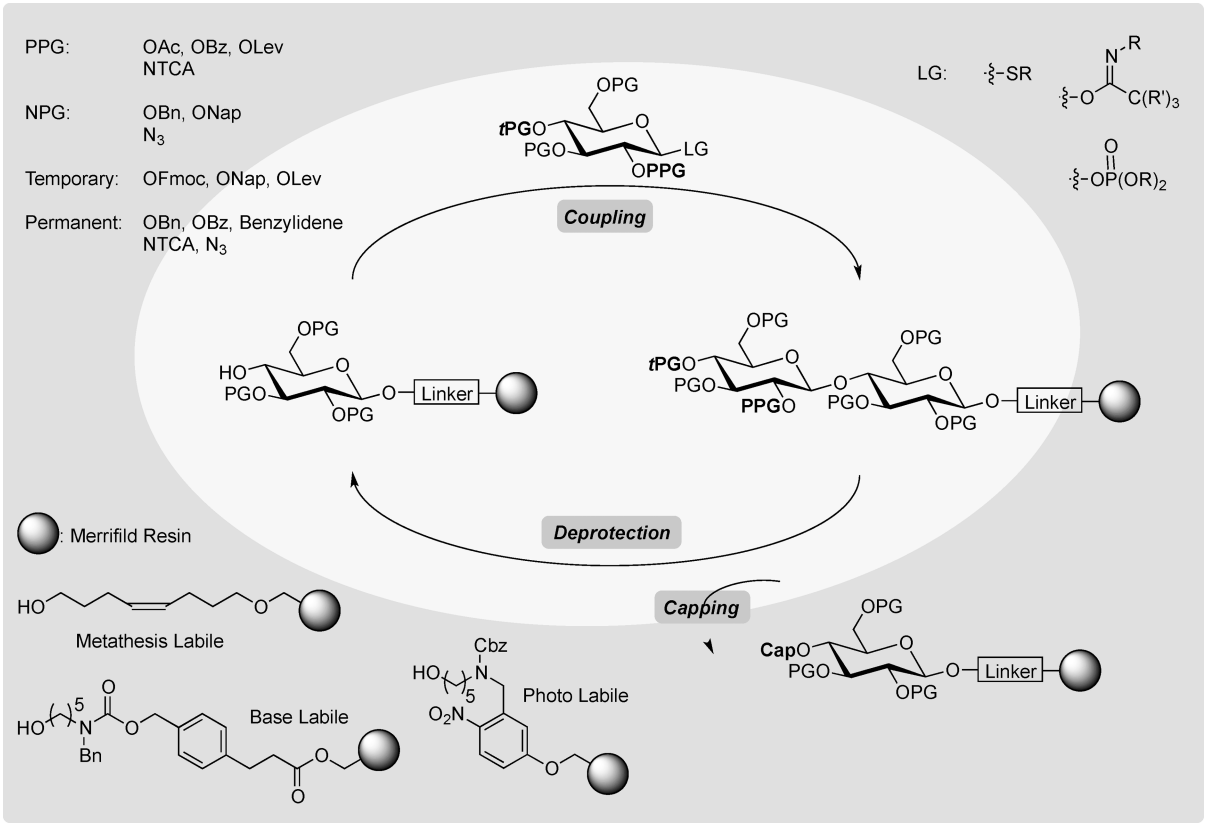

Figure 4. Overview over the coupling cycle and all strategic choices regarding protecting groups, linkers, and anomeric leaving groups.

and permanent protecting groups as well as anomeric leaving groups and activating conditions have to be compatible with the linker. Many of the fundamental considerations regarding strategy, resin, linker, and building blocks were explored initially by different groups that failed to combine the advances to a cohesive concept.[22]

The dizzying number of protecting groups has rendered synthetic carbohydrate chemistry particularly complex.[23] Ideally, automated glycan assembly relies on a minimal number of protecting groups that are electronically similar, are sterically not demanding, and have little or no conformational influence. We selected the benzyl ether as a permanent nonparticipating protective group for hydroxyls. Benzoate esters are used as participating permanent protecting groups for hydroxyl groups and replace the pivaloate esters that we used earlier since the removal of this group in larger glycans can be extremely challenging. The amine groups are permanently protected either as the participating *N*-trichloroacetate (TCA) or a nonparticipating azide protecting group. Similar to automated peptide chemistry, 9-fluorenylmethoxycarbonate (Fmoc) is used as temporary protecting group since it is readily removed by base and the dibenzofulvene cleavage product can be quantitated by UV absorption to estimate the coupling efficiency. Two orthogonal temporary protective groups are the participating levaloyl ester (Lev) cleaved selectively with hydrazine hydrate and the nonparticipating 2-methyl naphthyl (NAP) groups. Earlier, the acetate ester provided good results as temporary protective group[24] but more recently has been used to mark hydroxyl groups for sulfation during the synthesis of glycosaminoglycans.[25]

All among the wide variety of anomeric leaving groups that have been developed over the years and that can be activated reliably with Lewis acids can be used on solid support. Three types of glycosylating agents have been central to our effort: thioglycosides, which are very stable but react efficiently upon activation by electrophiles,[12] glycosyl phosphates,[13] which are activated by equimolar amounts of Lewis acids, and glycosyl acetimidates,[11] which require only catalytic amounts of Lewis acids.

Based on the strategic choice of building blocks including leaving and protecting groups, compatible building block–linker combinations had to be selected (Figure 4). Over the years we developed several linker systems that accommodate an increasing variety of protective and anomeric leaving groups: The octenediol linker **1**[26] can be cleaved by olefin metathesis and is compatible with a broad range of protective groups but cannot be used with thioglycosides since the electrophiles required to active the leaving group also attack the linker. The bifunctional linker **2**[27] is readily cleaved under basic conditions and releases conjugation-ready oligosaccharides that carry a aminoalkyl spacer at the reducing end and is compatible with all commonly used glycosylating agents. This linker does preclude the use of temporary acetyl ester protective groups such as acetates. The photocleavable linker **3** is most versatile and is cleaved in a continuous flow photoreactor.[25,28] Other groups developed different linkers for solid support synthesis but never used them for automated glycan assembly.

With a comprehensive synthetic strategy in place, each aspect of automated glycan assembly was addressed. The development process was not a linear progression but rather a gradual improvement in different aspects.

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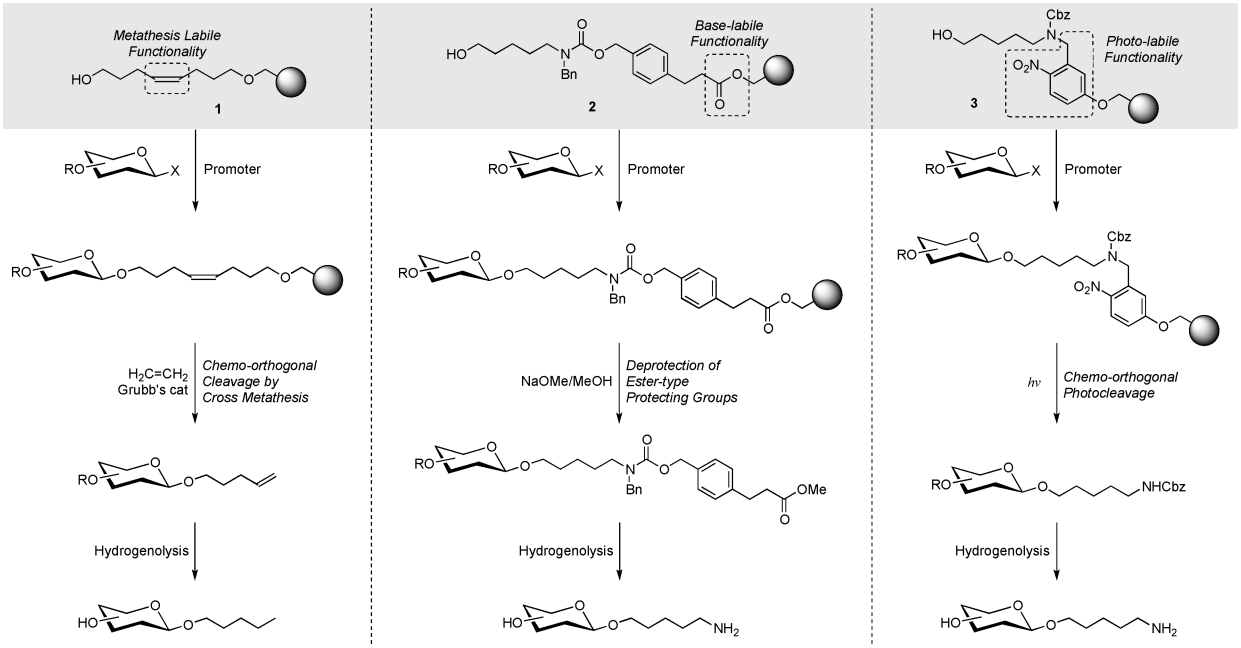

Figure 5. Linkers used for automated glycan assembly.

3. SOLID SUPPORT AND LINKER

The solid support that carries the growing oligosaccharide chain has a significant influence on the reaction conditions to be used during the assembly process. The experience with solid phase syntheses of oligonucleotides, peptides, and small molecules guided our choice of support matrix.[29] Polystyrene resins were found to be optimal considering compatibility with a broad range of solvents, robustness, loading capability, and prize.[26] Other resins and controlled pore glass (CPG) support gave acceptable results in many cases but suffered from lower loading, decreased mechanical stability, or retention of water in the polymer.

The choice of linker determines the reactions conditions to be tolerated during the assembly process. At the end of the synthesis, the linker has to be cleaved readily and efficiently to reveal the glycan. Among the many linkers developed for the solid support synthesis of oligosaccharides,[21] three modes of attachment are now commonly used for AGA (Figure 5). To capitalize on the fact that carbohydrates do not contain alkenes, an octenediol linker **1** was designed. Cleavage of this linker by virtue of the metathesis reaction in the presence of ethylene reveals a fully protected oligosaccharide *n*-pentenyl glycoside that can function as a glycosylating agent.[30] Octenediol linker **1** is compatible with most temporary protecting groups commonly used for oligosaccharide assembly but is not compatible with electrophilic reagents required to activate thioglycosides.[26]

Base labile bifunctional linker **2** was designed not only to link the oligosacchride to the solid support but also to reveal an alkylamine linker at the reducing end of the final product (Figure 5). Thereby, conjugation-ready glycans are produced that can be immediately coupled to proteins for vaccine development[31] or to surfaces in order to create glycan arrays.[32] The bifunctional linker is compatible with many protecting groups including Fmoc but not base labile temporary protecting groups such as acetate or benzyl esters.[27] With this linker, access to more complex glycans became possible (*vide infra*).

Automated glycosaminoglycan (GAG) assembly required one additional degree of protecting group orthogonality. GAGs such as chondroitin sulfate or heparin sulfate contain a multitude of sulfate groups. The hydroxyl groups to be sulfated following glycan assembly are best masked with ester groups that can be selectively removed in the presence of permanent ethers as has been shown for the modular assembly of heparin in solution.[33] Neither octenediol **1** nor the bifunctional linker **2** met the requirements, and a photocleavable linker **3** was designed.[25] Photocleavable linkers are in principle attractive and have been used for many applications including oligosaccharide synthesis previously.[34] However, cleavage efficiencies have been less than satisfactory because irradiation of solid support results in light scatter and variable reactions.[28] To improve cleavage efficiencies, a photochemical flow device consisting of a pump and transparent PFE-tubing wrapped around a mercury lamp was developed to ensure efficient cleavage of the linker and excellent recovery of the oligosaccharide product.[25] Our work on automated glycan assembly utilized the three linker systems **1**−**3** on polystyrene resin.

4. BUILDING BLOCKS

The glycosylation reaction is at the heart of glycan assembly (Figure 4). All regio- and stereochemical information is contained in the monosaccharide building blocks that are used for chain elongation. How many distinct monosaccharide building blocks would be required to prepare the entire mammalian or bacterial glycome? An analysis of the mammalian[35] and the bacterial glycan structures[36] contained in the glycan databases provided encouraging answers. Nature did not make all possible linkages and combinations; therefore, a rather small number of distinct building blocks is sufficient to prepare most mammalian *N*-glycans, *O*-glycans, and glycolipids.[35] Based on the frequency of a given glycosidic linkage in the database, the building blocks can be ranked (Figure 6). By applying the strategic protecting group and anomeric leaving

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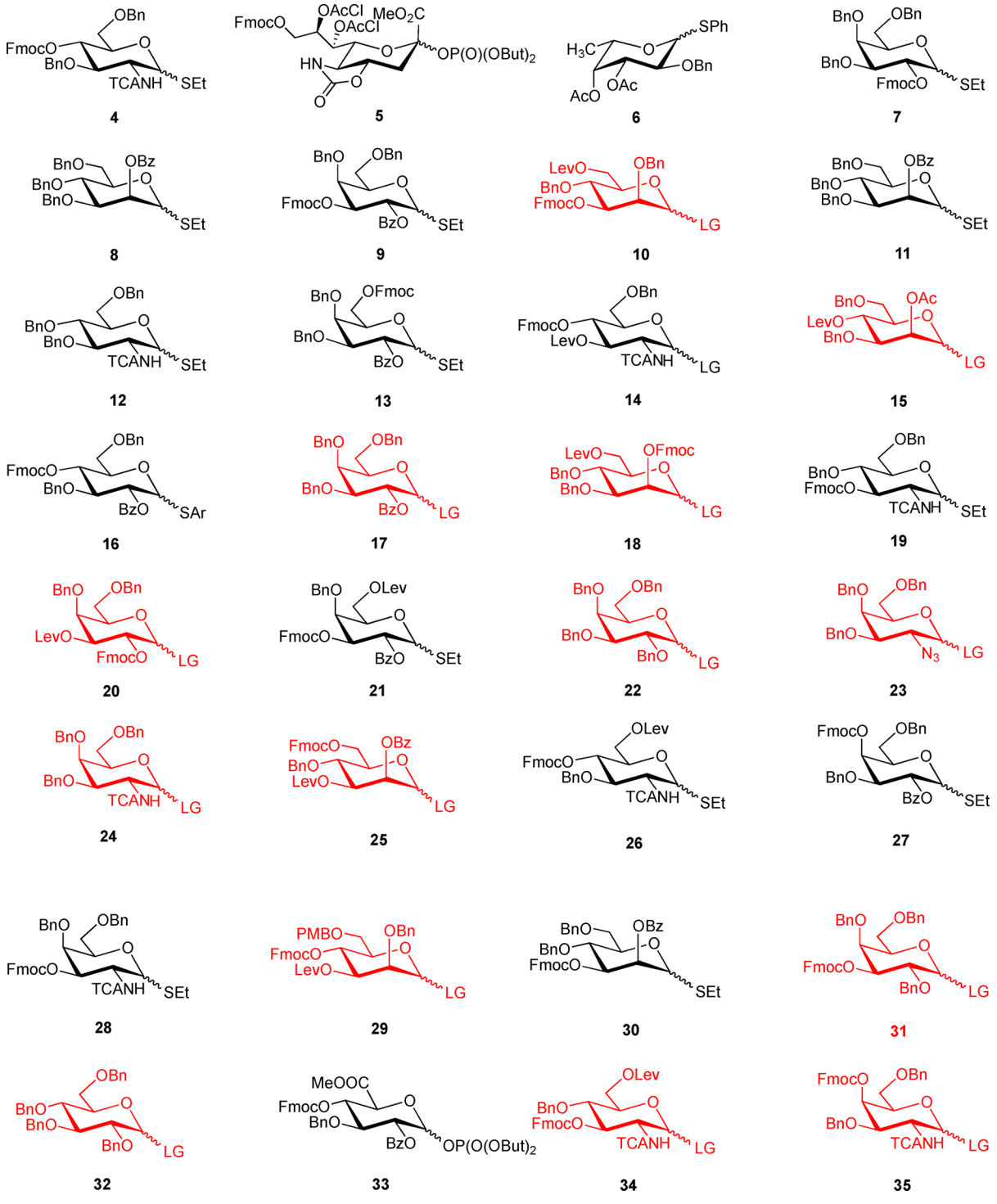

Figure 6. Building blocks for the assembly of mammalian glycans. The building blocks are sorted in decreasing prevalence based on bioinformatics analyses. Black indicates monomers already synthesized and used, while red building blocks have not yet been made.

group considerations discussed above, a set of building blocks was designed.[37]

The building blocks drawn in black (Figure 6) were prepared and utilized in automated syntheses, while some still await their synthesis and use (drawn in red). A set of "approved building blocks" that can be prepared reliably on large scale, are stable to storage for at least two months at −20 °C, and upon activation form the desired glycosidic linkage selectively in high yield has evolved. For each building block, key reaction parameters, such as activator, reaction temperature, and number of equivalents, have been determined. In contrast to one-pot procedures that rely on reactivity differences[15] for automated glycan assembly, a single building block suffices for the synthesis of homo-oligomers. Until very recently, synthetic access to the building blocks consumed almost 90% of our synthetic effort. With the commercialization of an increasing number of monomers, this effort has been greatly reduced. Still, the need for ever more complex glycans requires the synthesis of building blocks that cannot draw from isolated monosaccharides as starting materials.

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In case of the bacterial glycans, the diversity of required building blocks is much greater, but within each family of bacteria (e.g., actinobacteria), the number of required building blocks is less than ten.[36] An increasing number of a bacterial and plant monosaccharide building blocks has been prepared in the context of specific syntheses.

5. AUTOMATED SYNTHESIZER

During automated glycan assembly, the three step process of coupling, capping, and deprotection is executed by an instrument without operator interference. Initially, an instrument constructed for peptide synthesis was adapted by addition of a reaction vessel that allows for temperature adjustments.[38] Over the past 13 years, several generations of home-built systems were refined to eventually result in a commercial system, the Glyconeer 2.1 (Figure 7).[39]

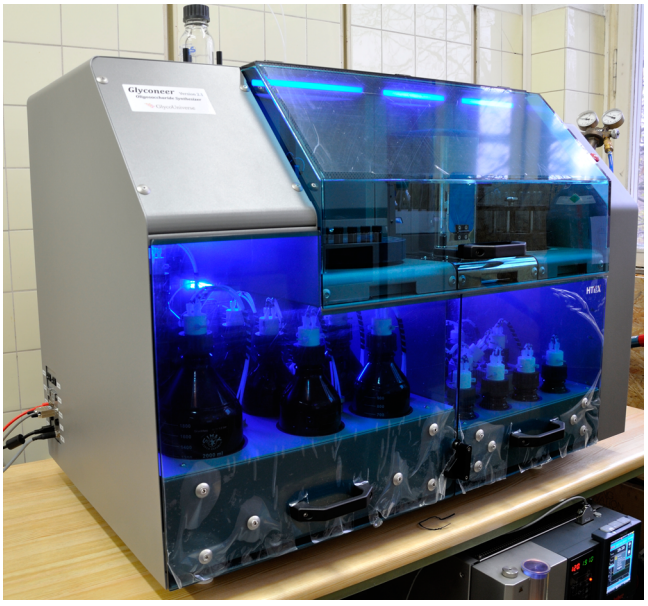

Figure 7. First commercial automated oligosaccharide synthesizer, Glyconeer 2.1.

This pressure-driven system draws building blocks from a carousel and reagents from reservoirs that are kept under an inert gas atmosphere. The jacketed glass reaction vessel is connected to a cryostat that allows for temperatures to be adjusted to as low as −50 °C and as high as 50 °C. The solid support rests on a fritted glass filter in the reaction vessel, and mixing is accomplished via an inert gas stream that is introduced from the bottom of the filter. The outlet of the reaction vessel can be directed to waste or to a fraction collector to recover excess glycosylating agent. Coupling efficiencies can be quantitated with the help of an UV sensor that detects the release of the temporary Fmoc protective group as is customary for automated peptide syntheses. Incorporation of each monosacccharide building block is achieved employing a coupling cycle that consists of a glycosylation reaction, several washing steps, removal of the temporary protecting group, and another round of washing steps. The computer program instructs the automated synthesizer to open and close valves that facilitate reagent flow driven by inert gas pressure. The program adjusts temperature, coupling time, and excess of building block based on preprogrammed values.

6. AUTOMATED GLYCAN ASSEMBLY OF OLIGOSACCHARIDES

With an automated synthesizer, resin with linker, and a set of building blocks on hand, the task of the synthetic chemist is an analysis of the target glycan considering the conceptual framework (Figure 4). Monosaccharide building blocks are identified based on the sugar (glucose, galactose, etc.), the connectivity (1 → 3, 1 → 4, etc., or potential branching), and the anomeric composition (α- vs β-linkage). The presence of multiple branch points requires careful planning and selection of building blocks because only one arm can be constructed at one time unless the arms are identical. With the selection of the synthetic strategy, the linker and the building blocks are identified. Automated glycan syntheses on the Glyconeer 2.1 are routinely performed on 25 μmol scale using polystyrene resin with a loading of approximately 0.25 mmol/g of resin. The resin is loaded into the temperature-controlled reaction vessel and the building blocks are loaded onto the instrument in the order of appearance. Upon initiation of the software, all further steps are executed based on the program.

The synthesis of complex glycan targets of biological significance served as a testing ground for new linkers, building blocks, instrument, and methods (see below). Thus, in the early days of automated glycan assembly, the focus was on the assembly process, and typically fully protected oligosaccharides were analyzed to determine coupling efficiencies, selectivities, and aspects of the automation process. The coupling cycles that we developed initially were not optimized for either time, reagent, or building block use but were rather aimed at obtaining the final product.[26,27,38,40,41] All reaction parameters are currently being optimized as part of the synthesizer commercialization process. As the automated process became more reliable, the synthetic effort turned to the procurement of glycans to be used as molecular tools.[27]

7. POSTAUTOMATION OPERATIONS AND PURIFICATION

The conclusion of the automated glycan assembly process is the beginning of several postautomation transformations. Typically, cleavage of the oligosaccharide from the solid support is followed by purification of the partially protected oligosaccharide products, before the removal of remaining protective groups furnishes the glycan target. When modified glycans such as sulfated GAGs are targeted, selective partial deprotection on the support is followed by sulfation and global deprotection.[25,42] The postautomation protocols evolved significantly over time as more complex synthetic targets required a more streamlined and structured approach to workup and purification.

7.1. Cleavage of the Oligosaccharide Product from the Solid Support

Cleavage of the initially used octenediol linker **1** to release a fully protected *n*-pentenyl glycoside oligosaccharide by a metathesis reaction with Grubbs' catalyst and ethylene required up to 36 h, longer than the automated assembly process itself.[38] This lengthy cleavage was a severe limitation of the initial process. Treatment of the bifunctional linker **2** with the strong base sodium methoxide released a partially protected oligosaccharide within 2 h. During cleavage, all ester protective groups were also removed to obtain a partially protected oligosaccharide.[27] Photocleavable linker **3** is most versatile because it allows for the use of acetate esters to mark sites for

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sulfation but requires a more elaborate setup for cleavage. A slurry of the resin is pushed with a HPLC pump through the flow photoreactor (Figure 8), consisting of PTFE tubing

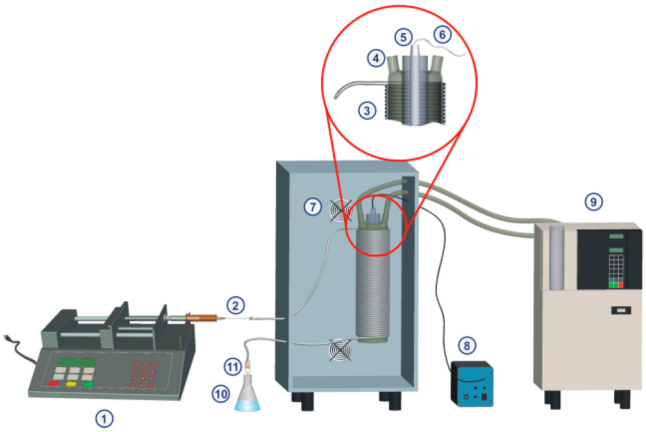

Figure 8. Continuous flow photoreactor for the cleavage of photolabile linker **3**: (1) syringe pump, (2) inlet, (3) tubing, (4) cooling system, (5) UV filter, (6) UV lamp, (7) fan, (8) power supply, (9) chiller, (10) outlet, and (11) frit.

wrapped around a mercury lamp,[25,28] to release a fully protected oligosaccharide. In unpublished work, commercial flow reactors for photochemistry have been successfully used to cleave linker **3** efficiently.

7.2. Analysis and Purification of Fully or Partially Protected Oligosaccharides

After cleavage from the resin, the crude product mixture obtained from the automated glycan assembly process is assessed by analytical HPLC. The conditions to be used for analytical HPLC depend on the linker system. Fully protected, hydrophobic oligosaccharides that are obtained when octenediol linker **1** or photocleavable linker **3** are used can be separated using normal phase columns. Following the analytical assessment, preparative HPLC is used to obtain the fully or partially protected oligosaccharides (Figure 5). Purification of partially or fully protected oligosaccharides is typically significantly easier than at the final stage of the deprotected, hydrophilic oligosaccharides that lack fluorescent groups. The protected oligosaccharides are fully characterized by ^{1}H and ^{13}C NMR as well as mass spectrometry.

7.3. Global Deprotection Followed by Purification of Conjugation-Ready Oligosaccharides

One or more chemical transformations are required to remove all protective groups and obtain pure oligosaccharides. Ideally, a single deprotection step using conditions that are simple to implement would be desirable. A simple process has not been reached yet except in some special cases.[43] When the base-labile linker **2** is employed, all ester protective groups are cleaved during release from the solid support such that only benzyl ether protective groups as well as any protecting groups on nitrogen remain.[27] Benzyl ether protective groups, as well as the Cbz-protection on the amine in the linker, can be removed by metal-catalyzed hydrogenation. To accelerate the removal of many benzyl ethers, high-pressure hydrogenations have become the method of choice for global deprotections.

When the octenediol linker **1** or photocleavable linker **3** are used, multiple cleavage reactions including strong base followed by metal-catalyzed hydrogenation may be required. Alternatively, Birch reduction using sodium in liquid ammonia is very effective, but these reaction conditions cannot be used by nonspecialists. Deprotection of larger oligosaccharides revealed that sterically hindered esters such as pivaloates are extremely difficult to cleave and prompted a redesign of monosaccharide building blocks to include benzoates in place of pivaloates.

The final fully deprotected oligosaccharide products have to be purified again. At this stage, the glycans are very hydrophilic and no longer contain any UV-active groups. Different means of purification have been explored over the years. Size exclusion chromatography is very effective to obtain high yields of completely pure oligosaccharides but is very time-consuming. A comprehensive evaluation of many column materials and eluent compositions yielded a broadly applicable and robust purification system. Both analytical and preparative HPLC give excellent separation when Hypercarb columns were used. The conjugation-ready oligosaccharides are characterized using NMR spectroscopy as well as mass spectrometry.

8. QUALITY CONTROL AND PRODUCT ANALYSIS

Synthetic glycans are characterized adhering to the rules established by the organic chemistry community to demonstrate identity and purity. Appropriate NMR (^{1}H, ^{13}C, and 2D NMR) experiments and mass spectrometry help to establish identity of the compound. To ascertain the stereochemical composition at every glycosidic linkage can be very difficult as oligosaccharides beyond ten residues are becoming synthetically readily accessible. The purity of organic molecules is typically established by elemental analysis. In the case of unprotected oligosaccharides, this technique reaches its limits since these molecules typically contain water, which adulterates the results. Consequently, the community has agreed to rely on NMR and mass spectrometry evidence to characterize the products of glycan syntheses. For the characterization of large oligosaccharides, at least 5 mg of final product are required even though for many biological experiments, such as for the production of glycan arrays, 1 mg of final product would be sufficient.

Efficient and reliable characterization of complex synthetic glycans will require new, sensitive methods that can distinguish between different anomers. Mass spectrometry is a powerful and highly sensitive method but, until very recently, could not distinguish stereoisomers. Very recently, we utilized a combination of ion mobility spectrometry and mass spectrometry (IM-MS) whereby the carbohydrate ions are not only separated according to their mass and charge but also the time that is required to drift through a region of the instrument that is densely filled with an inert neutral gas such as helium or nitrogen.[44] This additional dimension of information comes at no extra cost, because the sample and time requirements are similar to a conventional MS experiment. Moreover, the measured drift time can be converted into a collision cross section (CCS), an instrument-independent measure for the average area of the ion. IM-MS is of exceptional use for the structural analysis and quality control of complex, synthetically derived carbohydrates. Connectivity as well as configurational isomers can be separated efficiently with baseline resolution as demonstrated on trisaccharides, and the relative content of isomeric impurities can be determined quantitatively.[44] Since IM-MS instruments are commercially available, also nonspecialists are capable of performing such studies. If the method lives up to the early promise, a combination of HPLC and IM-

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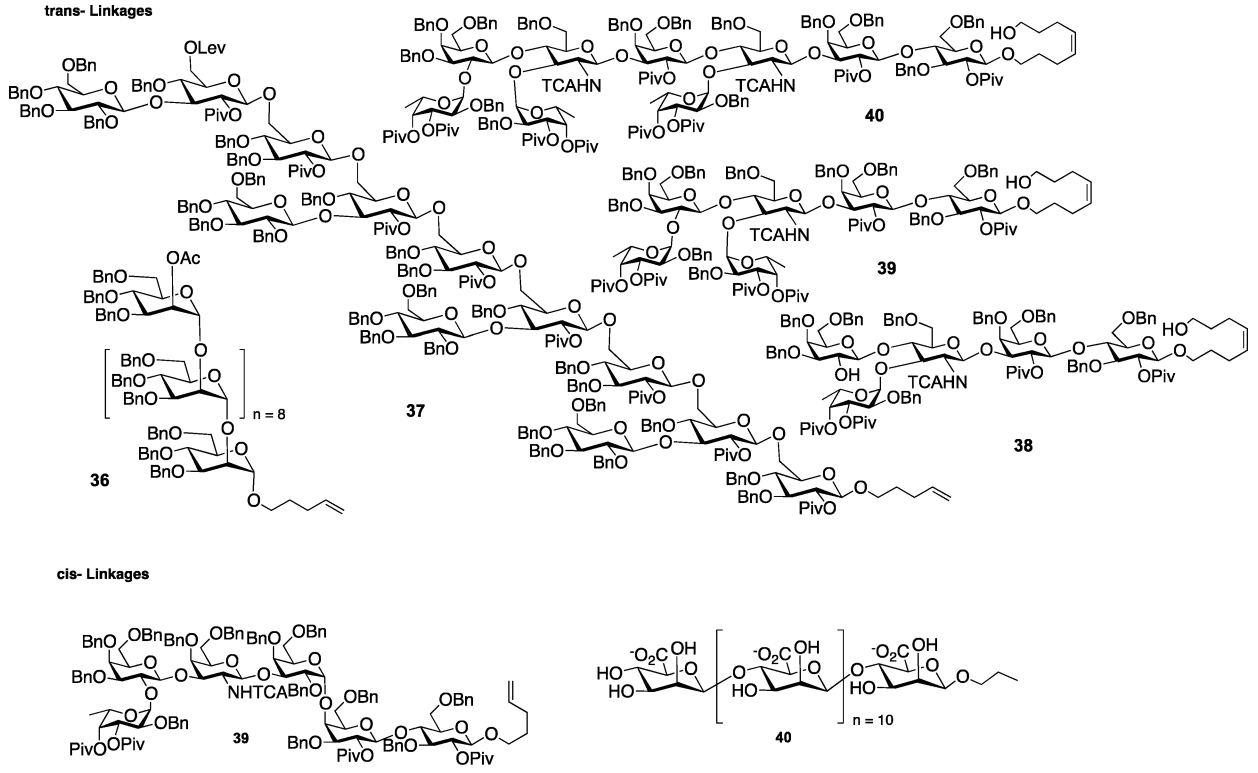

Figure 9. Automated glycan assembly of oligosaccharides with *trans*-glycosidic linkages (top) and *cis*-glycosidic linkages (bottom).

MS for purification and quality control of complex glycans using very small amounts of material will be possible.

9. CASE STUDIES FOR AUTOMATED GLYCAN ASSEMBLY

The procurement of complex glycans that serve as molecular tools for the glycosciences was the driving force of the methodological advances summarized above. Complex glycans served as a challenge for every aspect of the program ranging from strategic considerations to construction of an automated synthesis instrument. The refinement process resulted in increased efficiency of glycosylation reactions, as evidenced by the synthesis of chains as long as 30-mers,[43] and in access to a diversity of carbohydrates including GAGs[42] and glycopeptides.[45] Some key achievements in the refinement process to improve automated glycan assembly are briefly summarized below.

9.1. Oligosaccharides Containing *trans*-Glycosidic Linkages

Stereocontrol during glycosylation reactions is the most challenging part of every glycan synthesis. *trans*-Glycosidic linkages can be established with complete selectivity by virtue of participating protecting groups in the C-2 position (Figure 2). To explore the general aspects of automated glycan assembly such as synthetic strategy, resins and supports, as well as instrument development, initially homooligomers of α-(1 → 2) and oligomannosides were prepared.[26,38] These particular sequences were selected since the respective differentially protected monosaccharide building blocks are readily prepared in multigram quantities and result in high fidelity in the desired linkages. α-(1 → 2)Oligomannosides of varying lengths were assembled using the octenediol linker and a converted peptide synthesizer to demonstrate the viability of the automated glycan assembly concept.[26,38] A branched phytoalexin inhibitor oligosaccharide was constructed using disaccharide building blocks to install the branches while relying on a linear assembly strategy.[38] Blood group determinants and tumor-associated antigens served as examples for branched oligosaccharides that required the use of orthogonal temporary protective groups for elongation in two positions of a monosaccharide building block.[40]

9.2. Oligosaccharides Containing *cis*-Glycosidic Linkages

In contrast to *trans*-glycosidic linkages, *cis*-glycoside formation cannot rely on C-2 PPGs but depends on other factors. The anomeric effect ensures preferential albeit not exclusive formation of *cis*-glycosides such as α-galactosides. Automated assembly of the Globo-H hexasaccharide illustrated the formation of a *cis*-glycoside in the context of an oligosaccharide synthesis.[41] The most challenging situation is encountered in the case of β-mannosidic linkages where neither participating protecting groups nor the anomeric effect assist the synthetic chemist. Initially, the challenge was side stepped by using a disaccharide that already contained the difficult linkage.[46] As improved methods for the installation of β-mannosides were developed by others,[47] the respective building blocks were incorporated into automated syntheses.[27]

An impressive demonstration for the repeated installation of *cis*-glycosidic linkages is the automated synthesis of a β-(1,4)-mannuronic acid oligomer.[48] Polymers of β-(1,4)-mannuronic acid are a major component of the cell wall of various algae and the exopolysaccharide of *Pseudomonas aerigunosa*. Mannuronic acid building blocks are highly β-selective glycosylating agents as demonstrated for the synthesis of an oligomer (Figure 9).

9.3. Synthetic Glycosaminoglycans

GAGs are acidic, negatively charged polysaccharides that transduce extracellular signals to the interior of the cell.[49] GAGs are highly variable in size, ranging from 20 to 200

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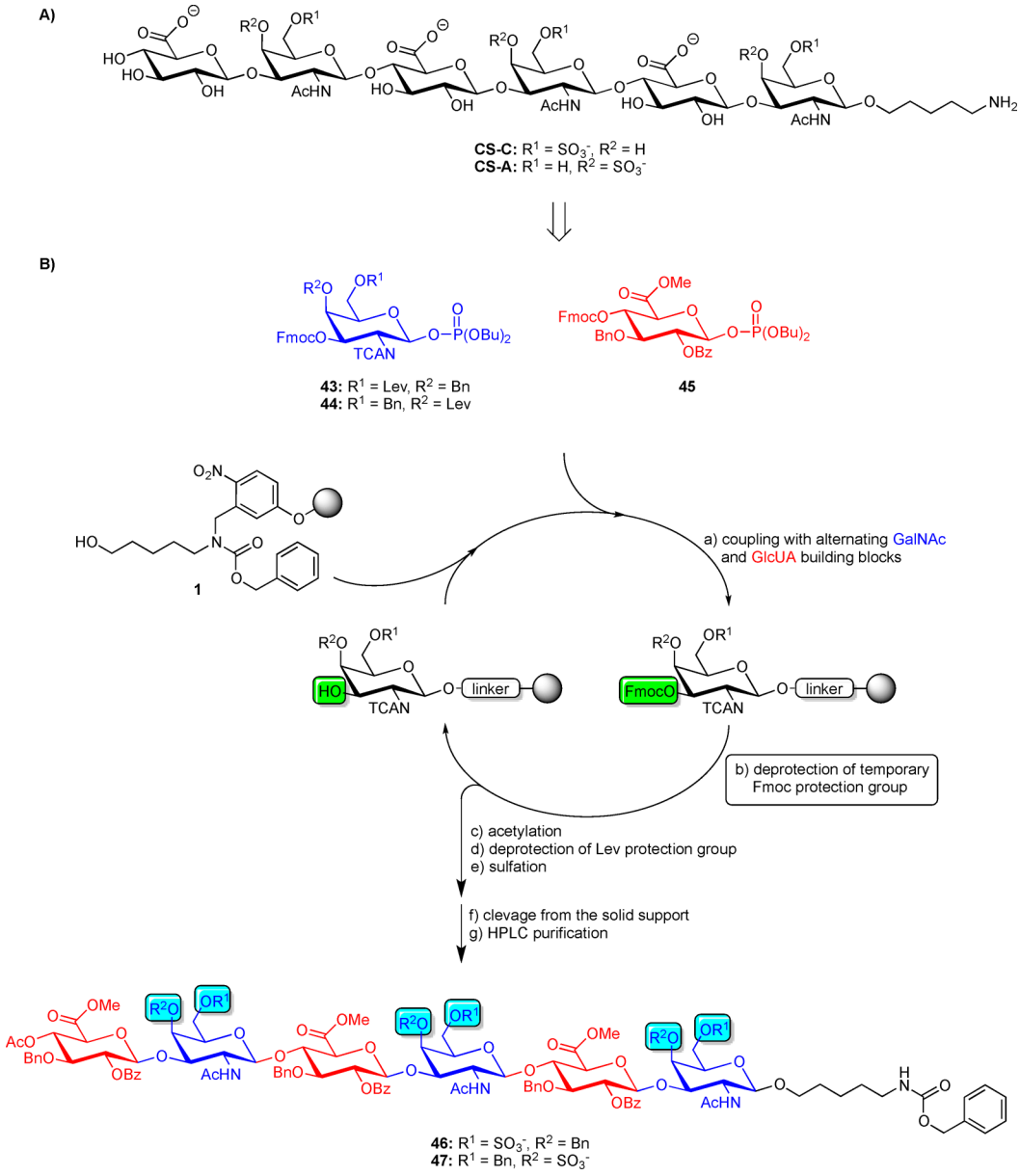

Figure 10. (A) Retrosynthetic analysis of chondroitin oligosaccharide sequences with different sulfation patterns. (B) Automated synthesis of chondroitin hexasaccharides. Reactions and conditions: (a) 3 × 3 equiv of building block, TMSOTf, DCM, −15 °C (45 min) → 0 °C (15 min); (b) 3 × 20% piperidine in DMF, 25 °C (5 min); (c) 3 × Ac_2O, pyridine 25 °C (30 min); (d) 3 × $H_2NNH_2{\cdot}H_2O$, pyridine, AcOH, DCM, 25 °C (60 min); (e) 3 × SO_3·pyridine, pyridine, DMF, 50 °C (3 h); (f) $h\nu$, DCM, 25 °C (**44**, 13% over 16 steps; **45**, 8% over 16 steps).

disaccharide repeating units, backbone composition, and the degree and pattern of sulfation. Chondroitin sulfate contains *N*-acetyl-*β*-D-galactosamine and *β*-D-glucuronic acid, and sulfation and acetylation of particular hydroxyl and amino groups vary. The sulfation patterns of GAGs influence the bioactivity of the molecules, but limited access to defined GAG structures has impeded mapping of structure−activity patterns.[50] A strategy for the modular solution phase synthesis of heparin oligosaccharides had been developed earlier[51] and guided the strategy for the automated synthesis: hydroxyl groups that were not modified during the reaction were permanently protected by benzyl ethers, hydroxyl groups that would ultimately be sulfated were masked with levulinoyl (Lev) esters that could be efficiently cleaved under mild conditions, and hydroxyl groups intended to serve as nucleophiles during glycan chain elongation were Fmoc protected. Two differentially protected galactosamine phosphate building blocks (**41**, **42**) and glucuronic acid phosphate building block (**43**) were combined on an automated synthesizer with a photolabile linker.[25] Release from the resin in a continuous flow photoreactor enabled the synthesis of two chondroitin hexasaccharides, **44** and **45** (Figure 10). Using this methodology with appropriate building blocks enabled access to other defined GAG oligosaccharides such as dermatan sulfate in a fraction of the time previously required.[42]

9.4. Synthesis of Sialylated Oligosaccharides

Sialic acid belongs to a family of nonulosonic acids, monosaccharides composed of a carboxylic acid nine-carbon backbone that play a unique role in glycobiology. Sialic acid-containing glycans mediate pathogen invasion[52] and are involved in signaling cascades.[53] Chemical sialylation is a significant challenge and usually plagued by low yields and

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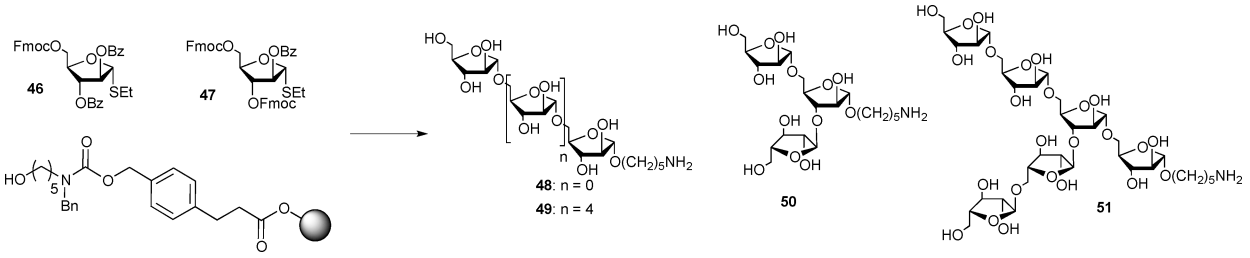

Figure 11. Automated assembly of oligoarabinans.

anomeric mixtures.[54] To avoid synthetic complications, sialic acid has often been introduced by enzymatic methods.[55] In naturally occurring *N*- and *O*-glycans, the terminal sialic acid residue is most often connected to the C3 or C6 hydroxyl group of galactose. To avoid problems associated with low yielding chemical sialylations, sialyl α-(2−3) and α-(2−6) galactosyl imidate disaccharide building blocks have been used for automated glycan assembly of several several sialic acid-containing oligosaccharides.[56] Very recently, we demonstrated that α-(2−6) sialylated glycans can be produced using monomer building blocks, albeit in relatively low yield.[57] Alternatively, the glycan backbone can be assembled on the synthesis instrument, and the sialylation is performed using enzymatic methods.[58]

9.5. Synthesis of Bacterial Oligosaccharides

Mammalian glycans are composed of just ten different monosaccharides. Bacteria rely on a much greater number of monosaccharides.[36] Access to the less frequent bacterial monosaccharide building blocks in differentially protected form required for automated glycan assembly is complicated by the fact that these monosaccharides are not available as starting materials for building block synthesis. Consequently, access to differentially protected monosaccharide building blocks often has to start from noncarbohydrate precursors via *de novo* synthesis. The *de novo* synthesis can rely on purely synthetic means,[59] or it can incorporate enzymatic steps.[60]

Arabinogalactan and lipoarabinomannan polysaccharides are major components of mycobacterial cell envelopes and contain multiple arabinose residues.[61] Arabinogalactan consists of three identical oligoarabinosides attached to a galactofuranose. Each oligoarabinoside contains arabinofuranose residues, linked together via α-(1−5), α-(1−3), and β-(1−2) glycosidic linkages. Synthetic oligoarabinofuranosides have proven to be useful tools for studying the biosynthetic enzymes involved in the mycobacterial cell wall synthesis.[62] Linear and branched conjugation-ready oligoarabinofuranosides (**48**−**51**, Figure 11) were assembled via automated glycan assembly.[63] Thereby, different arabinogalactan fragments for biological studies were procured.

9.6. Synthesis of Plant Oligosaccharides

The cell walls of all higher plant cells are surrounded by a polysaccharide-rich matrix.[64] Celluloses are the main components of plant cell wall polysaccharides. Xylan, a common hemicellulose, varies between plant species, but all possess a common backbone consisting of β-1,4-linked D-xylopyranoses. This backbone structure may be partially acetylated and substituted with L-arabinofuranosyl or D-(4-*O*-methyl) glucuronyl residues. Automated glycan assembly enabled rapid procurement of a collection of oligosaccharide fragments of plant arabinoxylans.[65] In the context of the synthesis of these plant glycans, the 2-methylnaphtyl group (Nap) as the first arming temporary protecting group was introduced to automated glycan assembly.

10. APPLICATIONS FOR GLYCANS ACCESSED BY AUTOMATED GLYCAN ASSEMBLY

Automated glycan assembly is not merely an intellectual exercise but rather strives to meeting the demand of glycoscientists for synthetic tools. Glycan arrays created by robotically printing synthetic glycans onto surfaces[66] for covalent attachment have become a standard tool for glycobiologists.[32] Today, carbohydrate content of glycan arrays is the decisive factor since all other aspects of the technology have been standardized. Access to pure glycans that can be covalently attached to a surface is the limiting factor. Automated access to conjugation-ready glycans is rapidly increasing the number and the diversity of glycans to populate arrays.

In addition to flat surfaces, glycans can be attached to nano- or microparticles or to fluorescent or radioactive tracers.[67] These glycosylated particles and surfaces can serve as probes to detect glycan−protein interactions,[68] to trace the fate of glycans in living animals, and to explore targeted delivery applications.[69]

Glycoconjugates that are created by attaching a synthetic glycan to a carrier protein enable several applications. Using glycoconjugates, monoclonal antibodies against glycans can be generated that serve as research tools to detect specific glycans in biological probes or even to create detection kits to sense biowarfare agents such as anthrax bacteria.[70] The most important application of glycoconjugates containing synthetic oligosaccharides is likely the development of semisynthetic vaccines against infectious diseases.[71]

11. REMAINING CHALLENGES FOR AUTOMATED GLYCAN ASSEMBLY

The fundamental aspects of automated glycan assembly have been established, and a variety of glycans of increasing length and complexity are now accessible quickly and reliably. With the advent of a commercial synthesizer and commercial reagents, many of the most time-consuming tasks for the synthetic chemist have been eliminated, in particular the synthesis of building blocks. A general strategy exists and the boundary conditions (protecting group pattern, anomeric leaving group) for the development of new approved building blocks are established. Better building blocks for the sialylation of glycans are urgently needed. More monosaccharide building blocks found in bacteria and plants will be required and will keep chemists busy for many more years. In order to create ever longer glycans, reaction conditions will require optimization to further improve yields. At the same time, a systematic optimization of the coupling cycles to minimize the use of building blocks and accelerate the coupling cycles will be

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required. The conceptual foundation has been laid, but additional work is required until a general solution similar to peptide or oligonucleotide synthesis has been reached.

12. CONCLUSIONS AND OUTLOOK

Automated glycan assembly has progressed from a concept 20 years ago to a commercially available technology. The solid support including different linkers, the monosaccharide building blocks, and the automated synthesis instrument are now commercially available. The automated assembly of oligosaccharide chains of increasing length (up to 50-mers in unpublished work) and more complex carbohydrates such as glycosaminoglycans and glycopeptides is now possible. Some building blocks such as sialic acids remain difficult to incorporate due to low yields, and combination of automated assembly and enzymatic sialylation has proven useful. Automated assembly of bacterial and plant glycans is possible, and representative examples have recently been disclosed. Key to the further development of the technology is the identification of more "approved building blocks" that function reliably and in high yield. Automated oligosaccharide assembly can utilize most techniques now available for glycan synthesis in solution; nevertheless, there are challenges remaining for organic chemists: novel methods for the efficient formation of challenging glycosidic linkages are needed to further expand the universe of accessible glycans. Automated glycan assembly provides important tools to advance the fundamental glycosciences and applications such as glycan array diagnostics and glycoconjugate vaccines.

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Notes

The authors declare the following competing financial interest(s): I have a significant ownership stake in Glyco-Universe GmbH & CoKG, the company that is commercializing the automated glycan assembly platform described in this Account.

Biography

Peter H. Seeberger studied chemistry in Erlangen (Germany) and completed his Ph.D. in biochemistry in Boulder (CO). After performing research at the Sloan-Kettering Cancer Center Research in New York, he built an independent research program at MIT where he was promoted to Firmenich Associate Professor of Chemistry with tenure after just four years. After six years as Professor at the Swiss Federal Institute of Technology (ETH) Zurich, he assumed positions as Director at the Max-Planck Institute for Colloids and Surfaces in Potsdam and Professor at the Free University of Berlin. His research interests span from synthetic chemistry to the glycosciences, immunology, and the development of diagnostics and vaccines.

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ABBREVIATIONS

AGA, automated glycan assembly; Cbz, carbyloxybenzyl; CCS, collision cross section; Fmoc, 9-fluorenyl-methyloxycarbonate; GAGs, glycosaminoglycans; HPLC, high performance liquid chromatography; IM-MS, ion mobility spectrometry and mass spectrometry; Lev, levaloyl ester; NAP, 2-methylnaphtyl group; NMR, nuclear magnetic resonance; NPG, nonparticipating protecting group; PCR, polymerase chain reaction; PPG, participating protecting group; TCA, trichloroacetate; UV, ultraviolet

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