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Perspective

Automated carbohydrate synthesis as platform to address fundamental aspects of glycobiology—current status and future challenges

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Abstract—This award account attempts to define the status of automated carbohydrate synthesis and its applications while trying to identify areas critical for further development. In this context the work of the Seeberger laboratory over the past 10 years is reviewed. Advances and shortcomings of the first automated oligosaccharide synthesizer platform will be discussed. Using this method, access to a multitude of complex oligosaccharides has been accelerated more than 100-fold. The synthesis of usable quantities of oligosaccharides has given rise to tools that had been common-place in nucleic acid and protein biochemistry. Carbohydrate microarrays are a versatile screening platform, and affinity columns and labeled carbohydrates are beginning to aid glycobiologists. While much has been achieved, many questions remain before a generally applicable set of tools will be available to facilitate carbohydrate research much in the same way oligonucleotide and peptide biology is explored today. Application of this technology to synthetic carbohydrate antigens in synthetic vaccine candidates against parasites and bacteria is attractive and has already yielded important insights.

Keywords: Oligosaccharide synthesis; Solid-phase synthesis; Automated synthesis; Synthetic carbohydrate vaccines

1. Introduction: the general idea

Scientific questions related to the structure and function of carbohydrates are complicated to address since defined oligosaccharides are difficult to produce.[1] Isolation from natural sources is lengthy, and very small quantities of materials that may contain more than one molecular species are obtained. The fact that the analysis of these isolated sequences is often complicated does not simplify the problem. Synthetic access to shorter oligosaccharide sequences is possible, but requires considerable skill. Over the past 100 years, synthetic chemists have invented new methods and improved these protocols to the point where oligosaccharides that are representative of glycoproteins, glycolipids, glycosaminoglycans and glycosylphosphatidylinositol (GPI) anchors can be prepared. An oligosaccharide synthesis remains for the most part a research project in itself and far from a routine operation. Accordingly, few synthetic carbohydrates are produced by any one laboratory, and many research groups specialize in particular oligosaccharide subclasses.

The way oligosaccharides are made today is reminiscent of peptide chemistry in the 1950s. At that time, using solution-phase couplings, ever more complex peptides and eventually even proteins such as insulin were prepared.[2] The advent of solid-phase synthesis developed by Merrifield in 1963 ushered in an era when access to most peptides has become a routine task that is often outsourced to service companies.[3] Access to defined

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oligonucleotides by total synthesis was a major undertaking for synthetic organic chemists, and the first gene was prepared in 1968 in a herculean effort by the Khorana group.[4] Fine tuning of the protecting group and phosphate ester coupling chemistries resulted finally in an automated synthesis method in 1980. Caruthers and co-workers established the solid- phase synthesis approach that now produces millions of short oligonucleotide sequences used as 'primers' for molecular biology experiments.[5] The automated solid-phase synthesis has been optimized to assemble even entire genes.[6]

From today's vantage point that benefits from hindsight, it may seem peculiar, but automated peptide and oligonucleotide synthesis were rather heavily criticized initially. The use of excess amounts of building blocks that called for as much as 20-fold excess of monomers that were the product of multistep syntheses appeared wasteful and overly expensive. Purification only at the end of the synthesis rather than after each step was feared to result in impure products and ambiguous biological results. High-performance liquid chromatography (HPLC) greatly helped to alleviate these worries. Still, some oligomer sequences remained difficult or impossible to construct using the automated methods. Only years after the proof-of-principle studies, access to these 'difficult' sequences became routine. In peptide assembly to this day, 45 years after the invention of solid-phase synthesis, such difficult sequences remain. Automated synthesis is broadly practiced even though some oligomers are difficult to prepare.

Automated assembly of oligopeptides and oligonucleotides totally changed the way the biology of these classes of biomolecules was investigated. Quick access to native structures and their analogs helped to establish structure–activity relationships. The synthetic molecules fill the toolbox that includes microarrays, affinity columns, labeled peptides, and oligonucleotides. Important scientific challenges related to peptides and oligonucleotides shifted from their synthesis to questions as to how a specific sequence may influence the function in a complex biological system. Fundamental medical questions and applications became the center of attention.

In the area of carbohydrate research, we are today in a situation that is reminiscent of the 1950s for peptide science and the 1970s for oligonucleotide research. We know that the different classes of carbohydrates are of great importance for biological processes and are medically highly relevant.[7] Some carbohydrates such as heparin and acarbose have become drugs, but are obtained by isolation rather than synthesis for the most part. Typically, mixtures of molecules rather than single molecular entities are employed. Projects such as the development of Fondaparinux®, a synthetic heparin pentasaccharide, relied on heroic efforts that required many years.[8] In the field of glycobiology, many results are difficult to interpret, since the exact structure of the active carbohydrate is not known. Tools that are common-place for the other two classes of biomolecules are nonexistent or have to be custom made by chemists. Access to these tools is highly limited.

Rapid and reliable access to oligosaccharides that are representative of all classes of glycoconjugates for any researcher interested in a biological or medical problem would completely change glycosciences. Defined molecules would give rise to tools that help elucidate the involvement of a carbohydrate in a biological event. Complex sugars also may simply be used for their structural features. The challenge to create a manageable, general synthetic method is the key to unleash a new wave in the glycosciences—similar to those bursts seen in the peptide and oligonucleotide fields decades ago.

2. Carbohydrates are complex—but how complex are they really?

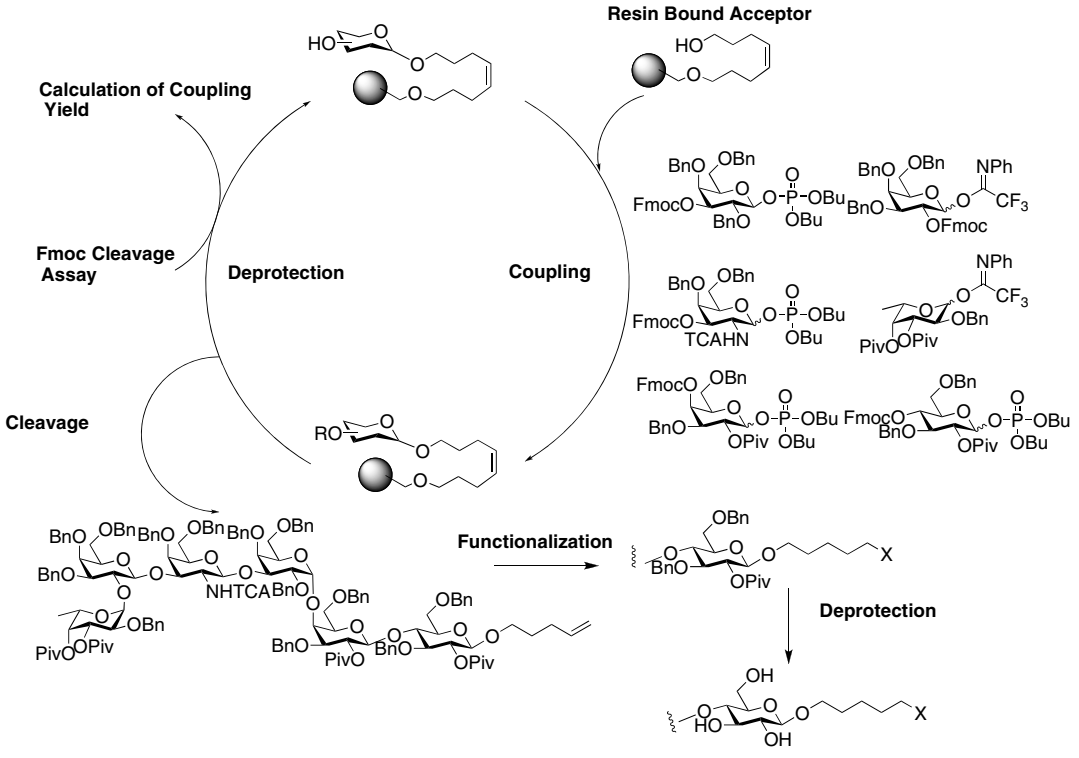
Comparing carbohydrates to peptides and oligonucleotides is not entirely fair. The latter two classes of molecules are strictly linear. Peptides are posttranslationally modified, but lipidation and glycosylation were ignored for the most part during the early days of peptide synthesis. Oligonucleotides are truly linear and just made up of four building blocks. The situation for carbohydrates is drastically different. First, the term carbohydrates really encompasses several classes of molecules that all exist as conjugates with other structures—glycolipids, glycoproteins, glycosaminoglycans (linked to proteins), and GPI anchors (combined with lipids and proteins). Ten monosaccharides are incorporated into these chains or polymers in mammalian systems, but many more monosaccharides are found in bacteria and other organisms. More basic building blocks, combined with the fact that oligosaccharides are branched and each glycosidic linkage constitutes a stereogenic center, theoretically results in an immense complexity of carbohydrate structures. If nature really occupies this entire glycospace, attempts by carbohydrate chemists to prepare a sizeable fraction of these molecules would be hopeless. Surprisingly, the question of what portion of the glycospace (the number of theoretically possible structures) is actually occupied by naturally occurring structures was not asked until last year. A definitive answer is of course impossible to give, but bioinformatics approaches using the most comprehensive databases of carbohydrate structures provided first insights.[9] Focusing initially on just mammalian N-glycans, O-glycans, and glycosphingolipids, it turned out that the structural space actually occupied is rather limited. Thus, around 40 appropriately protected monosaccharides are sufficient to construct most naturally occurring carbohydrates. While the number of required building blocks to construct bacterial sugars as well as glycosaminoglycans may further increase the number of

required monomers, overall a rather limited number of monosaccharide building blocks will be sufficient to construct many of the desired sequences. This realization is important when contemplating the systematic chemical synthesis of many oligosaccharides.

3. Automated synthesis—challenges and solutions

The automated solid-phase synthesis of small quantities of peptides and oligonucleotides has proven superior to any other method in terms of speed and convenience. Thus, an analogous approach for oligosaccharides appears attractive. First attempts at solid-phase oligosaccharide synthesis in the 1970s were futile due to the lack of efficient glycosylation methods amenable to the solid-phase paradigm.[10] After a period of dormancy in the 1980s, the next decade saw renewed interest in solid-phase carbohydrate chemistry. A host of studies exploring different solid support materials, linkers, synthetic strategies, and glycosylating agents appeared. Still, a comprehensive solution to the challenges of automated oligosaccharide synthesis remained elusive.[11]

Starting in 1998, our laboratory systematically studied all aspects of automated solid-phase synthesis (Scheme 1). Employing the acceptor-bound approach that fixes the nucleophile on the solid support, we selected Merrifield's polystyrene resin as the solid support for its stability, swelling properties, and availability. A key strategic decision was the choice of a linker that has to remain stable during the coupling and deprotection events, but is to be readily cleavable following the synthesis. Ideally, linker cleavage will leave a spacer on the reducing end for further functionalization. For this purpose we selected a 4-octenediol linker that can be cleaved via olefin cross-metathesis in the presence of Grubbs' catalyst and ethylene to release a fully protected oligosaccharide in the form of an *n*-pentenyl glycoside.[12] Glycosyl phosphates and glycosyl trichloroacetimidates, a staple of carbohydrate synthesis, served as glycosylating agents. In order to drive coupling reactions to completion and ensure high coupling yields, 5 equiv of the building block was added twice to the resin. Use of excess building blocks, while painful during the initial stages when these monomers are not yet commercially available, was a common problem for both peptide and oligonucleotide synthesis. The custom-designed cooled reaction vessel allowed for the adjustment of the reaction temperature during coupling reactions to temperatures as low as −15 °C. This temperature was low enough for many efficient couplings, but not for the activation of some of the powerful glycosylating agents. Acetate or levulinoate ester and 9-fluorenylmethoxy carbonyl (Fmoc) groups served as temporary protective groups that had to be removed following each coupling. Fmoc removal can be monitored by UV spectroscopy, a common procedure during peptide assembly. Selective cleavage of these ester or carbonate groups exposed the hydroxyl group that was to serve as the nucleophile in the next coupling. Placement of



Scheme 1. Automated solid-phase oligosaccharide synthesis overview.

two orthogonal protective groups on one building block resulted in the introduction of branched oligosaccharides.[12,13] Coupling cycles consisting of coupling and deprotection steps were repeated until the desired oligosaccharide was assembled. Typical cycle times required between 90 and 180 min for the addition of one building block to the growing oligosaccharide chain.

Cleavage of the fully protected oligosaccharide from the solid support was achieved by a cross-metathesis reaction in the presence of ethylene. This cleavage reaction required relatively high catalyst loadings (up to 20 mol %) and reaction times of one to two days. The recovery of the *n*-pentenyl oligosaccharides was often excellent, but varied in some instances. Removal of all protective groups by Birch reduction using sodium metal dissolved in liquid ammonia relieved the oligosaccharides of all masking groups, but did not touch the terminal double bond. This alkene moiety served as the attachment point for proteins or to surfaces following several chemical transformations.

Analysis and purification of the reaction products employed HPLC.[13] Deletion sequences and other unwanted byproducts of the assembly process were reliably separated. These purification protocols proved particularly important when cis-glycosidic linkages were prepared that could not rely on the aid of participating groups to control the stereochemical outcome of the glycosylation event that facilitates the formation of trans-glycosidic linkages.[14] HPLC served to separate the different stereoisomers formed during some of the most challenging linkages such as β-D-mannosides with relative ease.[15]

4. Automated synthesis—future challenges

This first automated oligosaccharide synthesizer has provided access to even more complex oligosaccharides that contain an increasing variety of linkages in typically less than one percent of the time required by traditional solution-phase syntheses. In about one day, the assembly process is complete, compared to processes that may require weeks to months. Nonetheless, the system we introduced has drawn sharp criticism from many carbohydrate chemists. These criticisms mirror the situation encountered by the pioneers of solid-phase peptide and oligonucleotide synthesis, and even the specific points raised by the critics are reminiscent of these earlier accomplishments:

4.1. Building blocks are used in excess

In the case of oligosaccharide assembly, as much as 5–10-fold excess is used, similar to peptide assembly, but less than what was required for oligonucleotide preparation. As long as these building blocks are not commercially available, their procurement constitutes the biggest time commitment for carbohydrate assembly. Experience has shown that once synthetic protocols are available and demand increases, commercial building block supply will follow.

4.2. Complete control over stereochemistry at each new anomeric carbon cannot be exercised

It would be desirable to maintain complete stereocontrol at each coupling step. We have demonstrated with several examples that purification at the end of the synthesis is sufficient to separate unwanted stereoisomers. The advent of HPLC was responsible for the breakthrough of automated peptide synthesis and is just as important for automated oligosaccharide synthesis.

4.3. Not every glycosidic linkage can presently be installed by automated synthesis

Ultimately, it will be necessary to introduce most glycosidic linkages efficiently by automated assembly. Initially, we are focusing on the constituents found in mammalian systems. Forty-five years after the invention of solid-phase peptide chemistry 'difficult' sequences remain. Likely, the situation for carbohydrates will be similar: many sequences will become routine while further innovation will be required to make the entire sequence space accessible.

4.4. Thioglycoside building blocks cannot be used

The double bond in the linker precludes the use of thioglycosides that necessitate electrophiles for activation. Thioglycosides are a staple of carbohydrate chemistry due to their ease of preparation and stability. A different linker will have to be developed to allow for thioglycosides to be incorporated.

4.5. Linker cleavage is slow

The cross-metathesis reaction that is used to cleave the linker is selective and efficient, but in many cases takes just as long as the assembly process itself. In addition, cleavage involves the use of a transition metal catalyst that may introduce impurities into the final product. New linker designs that allow for faster release from the resin under simpler reaction conditions need to be developed.

4.6. Linker functionalization requires additional steps

Following release of the fully protected oligosaccharide from the solid support in the form of an *n*-pentenyl glycoside, the remaining double bond is utilized to install a unique functional group that serves to attach the oligosaccharide to a chip surface, protein, or other carriers. A new linker design that would already incorporate a

spacer and a unique functional group to be exposed upon release from the solid support without the need for additional functionalization steps is needed.

4.7. Protecting group removal requires several steps

Currently, ester and other protective groups are used to mark the different functional groups of the oligosaccharide. Streamlined and simplified deprotection protocols that can be carried out by nonchemists will ultimately be needed. The area of functional group protection will require significant innovation.

4.8. The converted peptide synthesizer cannot automatically adjust to low temperatures, cannot be cooled to temperatures below −20 °C, and is not commercially available

A fully automated instrument that can automatically adjust reaction temperatures, without operator interference, can work with short coupling cycles, and achieve coupling temperatures as low as −70 °C will have to be developed. Availability of such a system at reasonable cost will be a key step toward wide acceptance of automated oligosaccharide synthesis.

Over the past 10 years, our laboratory has offered solutions to the challenges of automated oligosaccharide synthesis. Still, many important questions (vide supra) need to be addressed to make our approach usable for the nonspecialized chemist or even for a biologist. All we have shown to date is that automated oligosaccharide synthesis can be used to prepare most glycosidic linkages and assemble target structures about 500 times faster than was possible previously. Now it is time to address the problems described above and make the method generally available.

5. Synthetic oligosaccharides are key tools for glycobiology

Accelerated access to milligram amounts of pure, structurally defined oligosaccharides enables biologists and biochemists to tackle scientific questions that were previously difficult to address.[16] Efforts to define structure–activity relationships have relied on synthetic carbohydrates for decades. In cases where multiple rounds of experiments were required, chemical synthesis became prohibitively slow. Fast, automated methods have now begun to impact biology since more structures are now accessible in much shorter time. Accordingly, automated synthesis will ultimately provide the tools for glycomics investigations (Fig. 1).[17] Carbohydrate arrays have proven very powerful in determining the binding specificities of carbohydrate-binding proteins and antibodies, RNA and entire cells.[18] Following initial reports that focused on the technical feasibility of different approaches, attention in the past few years has shifted to applications to address biological issues. The utility of carbohydrate arrays is now only limited by the content to be placed on chip surfaces—accelerated synthesis will have to meet this increasing demand for complex structures.

In addition to attaching synthetic oligosaccharides to microarray surfaces, they can also be affixed to polymers or magnetic beads to create affinity columns. These columns have been used to isolate previously unknown carbohydrate-binding proteins of biological relevance. Ligation of oligosaccharides to fluorescent markers of highly fluorescent quantum dots creates functional tools for both in vitro and in vivo imaging. Adornment of dendrimers or polymers with defined oligosaccharides provides tools to investigate multivalency effects and potential inhibitors of specific carbohydrate–protein interactions.[19]

6. Toward synthetic carbohydrate vaccines

In addition to their use as mere research tools, synthetic carbohydrates hold many opportunities for use as diagnostics, vaccines, and drugs.[20] Any project that is currently relying on isolated carbohydrates can benefit from the advantages a defined molecule has to offer. Carbohydrate-based vaccines are an obvious area to employ synthetic oligosaccharides. Already, several carbohydrate-based conjugate vaccines are protecting millions

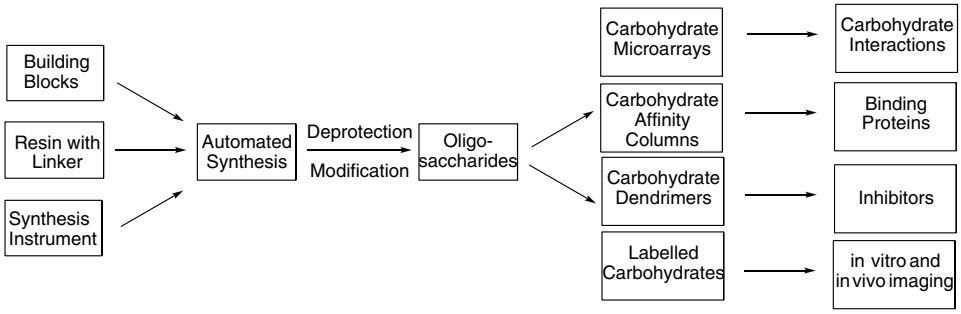


Figure 1. Carbohydrate synthesis and use of synthetic carbohydrates.

of children world-wide against bacterial infections caused by bacteria such as *Haemophilus influenza* type B.[21]

The concept is fundamentally as simple as it is efficient. A unique cell-surface carbohydrate that is only present on the surface of an infectious agent is identified, and the respective oligosaccharide is isolated from cultured bacteria. Chemical conjugation of the carbohydrate to a carrier protein is required to result in a construct that is recognized as foreign by infants and met with a significant immune response that produces antibodies to counteract the infectious agent.

Current carbohydrate-conjugate vaccines are biologicals, their production is relying on strictly controlled isolation and production processes that define the mixture of molecules to be included in the vaccine formulation. Since not all infectious agents can be cultured, and the carbohydrate antigens even of those that can be grown cannot always be efficiently isolated, synthetic chemistry provides an attractive alternative. Since it is not known what portion of a large polysaccharide is sufficient to serve as an antigen, vaccine development will typically require the synthesis of various oligosaccharide candidate antigens. Multiple rounds of synthesis help to establish a structure–activity relationship (SAR) and link a particular oligosaccharide antigen with a protective effect. Such syntheses, while possible, have been prohibitively time consuming. The breakthroughs in automated synthesis of ever more complex oligosaccharide antigens that now provide access to carbohydrate molecules much faster are beginning to impact vaccinology at several levels:

- The synthesis of defined oligosaccharides that can be conjugated to carrier proteins has been accelerated, and multiple rounds of SAR studies can be conducted rapidly.
- Carbohydrate microarrays containing complex structures serve to determine immune responses in human sera and to map antibody epitopes.

These technologies have begun to impact the development of a host of carbohydrate vaccine candidates in academic and industrial laboratories including our own. We are currently advancing molecules against malaria, leishmaniasis, a host of bacterial infections including biowarfare agents such as anthrax, fungal infections as well as viral candidates toward clinical trials (see Chart 1).

The case of the anthrax antigen on spores illustrates how straightforward the process can be: Following disclosure of the oligosaccharide structure found on *Bacil-*

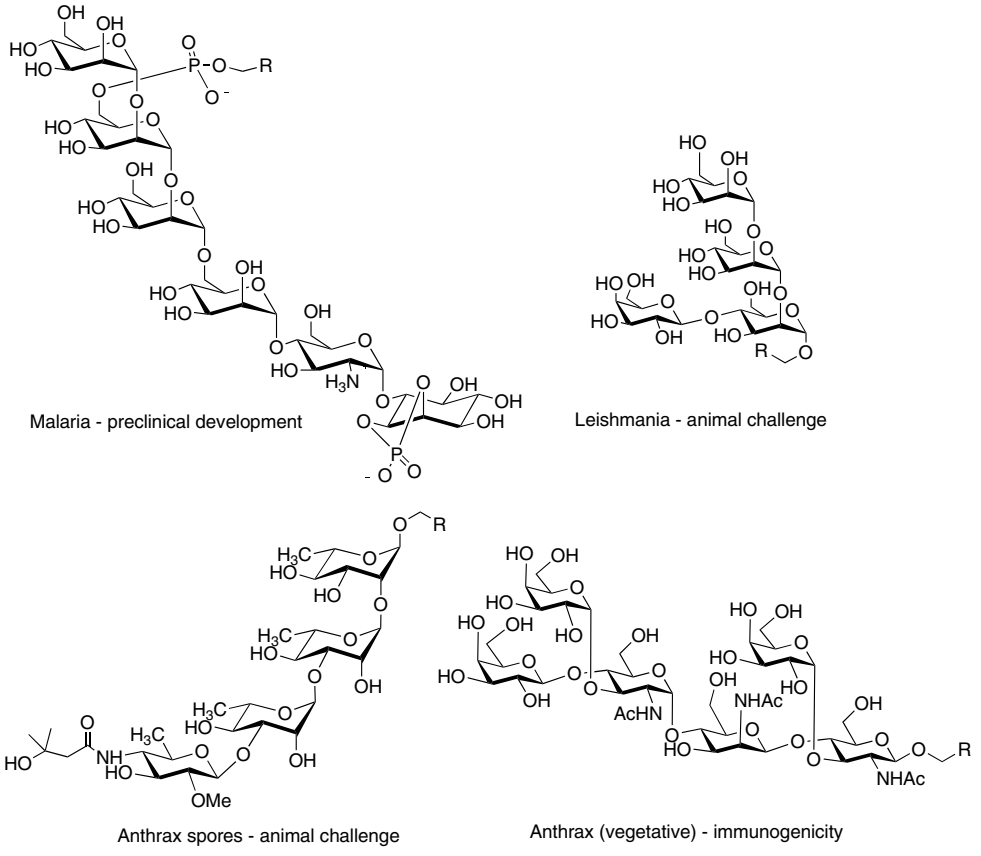


Chart 1. Some examples of synthetic carbohydrate antigens currently under development in the Seeberger laboratory.

lus anthracis spores,[22] the tetrasaccharide was assembled using chemical synthesis,[23] conjugated to a carrier protein, and monoclonal antibodies were produced.[24] These antibodies exhibit some cross reactivity with *B. cereus* and limit the utility of these antibodies as detection agents for anthrax spores. The tetrasaccharide–protein conjugate was found to be immunogenic in mice and is currently undergoing challenge studies.

In addition to anthrax, a wealth of other bacterial targets is currently being pursued. Around 10 different antigens found on the surface of bacteria that infect children or at-risk populations in the developed and developing world are proceeding toward clinical testing in our laboratory and a spin-off company, Ancora Pharmaceuticals (Medford, MA, USA).

These bacterial targets are relatively straightforward: it had been established previously that different bacterial strains carry unique oligo- or polysaccharide antigens that can serve as candidates for vaccine development.[25] The power of synthetic tools is even more vividly illustrated in the case of a malaria vaccine candidate. Based on initial experiments by Schofield using tiny amounts of isolated mixtures of GPI anchor glycolipids, it was proposed that glycans of this type may confer protection from death by *Plasmodium falciparum*, the parasite that causes malaria.[26] The exact glycan structure was not known, but a synthetic oligosaccharide, when conjugated to a carrier protein, helped to protect mice from a challenge with cerebral malaria.[27]An antitoxin effect rather than killing of the parasite provided protection. The parasite as well as the animals survived. Antitoxin vaccines are not without precedence as both diphtheria and tetanus vaccines operate on this principle.

Although the protective powers of the GPI-conjugate had been demonstrated, the fundamental mechanism of action was not known. Reports indicated that adults in endemic malaria areas are protected from severe disease via a resistance mechanism.[28] Mainly children under the age of two years are victims of cerebral malaria—an observation that may correlate with the inability of infants up to this age to form anti-carbohydrate antibodies. To test this hypothesis, a tool for high-throughput screening of thousands of human sera in parallel was needed. Ideally, a miniaturized assay would require minimal amounts of carbohydrate and serum. A carbohydrate microarray carrying defined GPI-glycan structures was introduced to screen sera from populations in different endemic countries as well as naïve individuals. The screening experiments revealed that a specific response against particular GPI-glycans protects adults in endemic areas from severe disease.[29] Children under two years of age and naïve individuals failed to show antibody titers. Some adults that took part in a challenge trial developed anti-GPI antibodies upon infection with *P. falciparum*. These studies illustrate the power of synthetic tools that were most recently brought to bear on the search for the biochemical mechanisms that underlie the action of GPI.

When anti-GPI-glycan antibodies provide protection from cerebral malaria, this sugar must be part of the chain of events that is the underpinning of malaria disease. We were able to identify a novel receptor on part of the human immune system. This receptor is also a key component in the life cycle of *P. falciparum* and blockage of the GPI-receptor interaction protects from malaria infection.[30] Identification of the receptors was only possible due to the availability of affinity columns containing synthetic GPI. Other tools such as polymers and dendrimer-containing GPIs were used as inhibitors, while GPI-containing quantum dots help to image some of the key molecular interactions.[30]

The studies that focus on malaria emphasize the advantages of rapid access to oligosaccharides, and the molecular tools that can be produced using these molecules hold. By bringing defined structures systematically to bear on disease processes that involve carbohydrates, active carbohydrates are identified. Detailed insights into biological recognition phenomena based on carbohydrate–protein interactions will result and provide targets for drug discovery.

7. Conclusions

The fundamental aspects of automated solid-phase oligosaccharide synthesis have been addressed, and the first automated oligosaccharide synthesizer has been introduced. Accelerated access to defined oligosaccharides provides now important research tools for glycobiology. In addition, synthetic carbohydrate antigens are beginning to make their way toward the clinic in a number of diseases including malaria as well as bacterial infections.

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