

CHEMICAL GLYCOMICS – FROM CARBOHYDRATE ARRAYS TO A MALARIA VACCINE

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ABSTRACT

Chemical glycomics uses synthetic chemistry to procure defined carbohydrate molecules to study the glycans involved in many functions in the living cell. Based on an automated synthesis platform, a host of synthetic tools including carbohydrate microarrays has been developed. These tools have been employed to dissect carbohydrate-copolymer interactions. Basic research in the glycomics arena is beginning to impact on drug discovery, especially the development of carbohydrate-based vaccines. The development of vaccine candidates to protect from malaria and leishmaniasis infections is discussed.

INTRODUCTION

Glycomics is defined in analogy to genomics as the entire set of glycans produced in a single organism. The realization that carbohydrates carry out important functions beyond energy storage and providing structural stability of the cell wall is not new, but the tools required to advance glycobiology at a more rapid pace were largely missing. Better, faster, more reliable and more sensitive sequencing techniques for most glycoconjugates have been developed in recent years [1]. Better sequencing allows for the comparison of carbohydrate structures between different cell populations and the identification of relevant carbohydrates even when those are present in very small amounts in a particular system. An improved molecular understanding of specific structures resulted in a dramatically increased need for defined molecules in quantities that are sufficient for biological, bio-

chemical and biophysical studies. Speedy access to defined carbohydrates in sufficient quantities is needed to gain access to oligosaccharides for the creation of tools that are commonplace in genomics and proteomics.

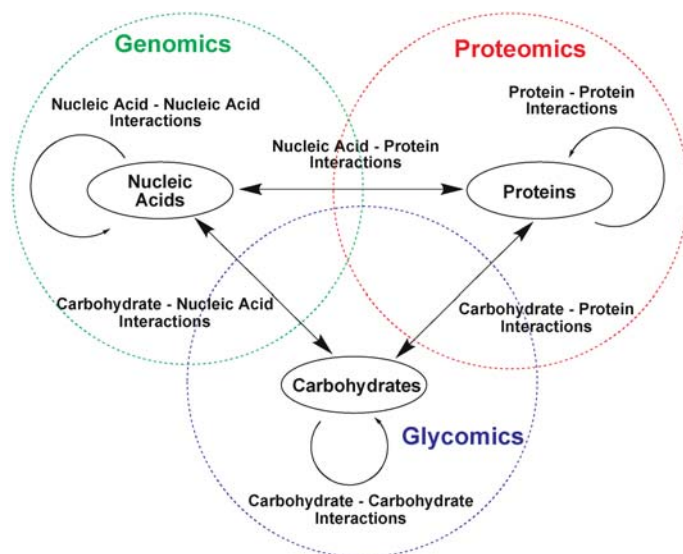


Figure 1. Interactions of the three main biopolymers. (Reprinted from [2] with permission of the RSC.)

Synthetic carbohydrates are needed to study carbohydrate-carbohydrate, carbohydrate-protein, and carbohydrate-nucleic acid interactions (see Fig. 1). Rapid advances in the field of glycomics have been hindered by the complexity of the biomolecules involved. Oligosaccharides are structurally more complex than nucleic acids and proteins due to their frequent branching and linkage diversity. The difficulty in isolating, characterizing and synthesizing complex oligosaccharides has been a significant challenge to progress in the field.

AUTOMATED SYNTHESIS OF OLIGOSACCHARIDES

Unlike the other major classes of biopolymers carbohydrates are often characterized by highly branched motifs. Each monosaccharide unit has multiple sites of attachment to the next sugar moiety. Additionally, each glycosidic linkage connecting two sugar units can take on one of two possible isomeric forms. There are over one thousand different trisaccharides possible when the ten mammalian monosaccharides are combined. The synthesis of oligosaccharides has been pursued for over 100 years and the key coupling, the glycosylation reaction is one of the most thoroughly studied transformations in organic chemistry [3]. The anomeric substituent acts as a leaving group thereby generating an electrophilic intermediate. Reaction of this species with a nucleophile, typically a hydroxyl group, leads to the formation of a glycosidic linkage. A host of anomeric leaving groups has been utilized in the construction of oligosaccharides. In addition to the formation of the glyco-

sidic linkage, the multitude of functional groups (amino and hydroxyl) of similar reactivity on each monomer emphasize the need for effective differentiation to allow for access to branched structures. A plethora of protective groups for the masking of amino and hydroxyl groups has been introduced. While much progress has been made, some linkages still remain difficult to install. Particularly the synthesis of large, branched oligosaccharides presents difficulties.

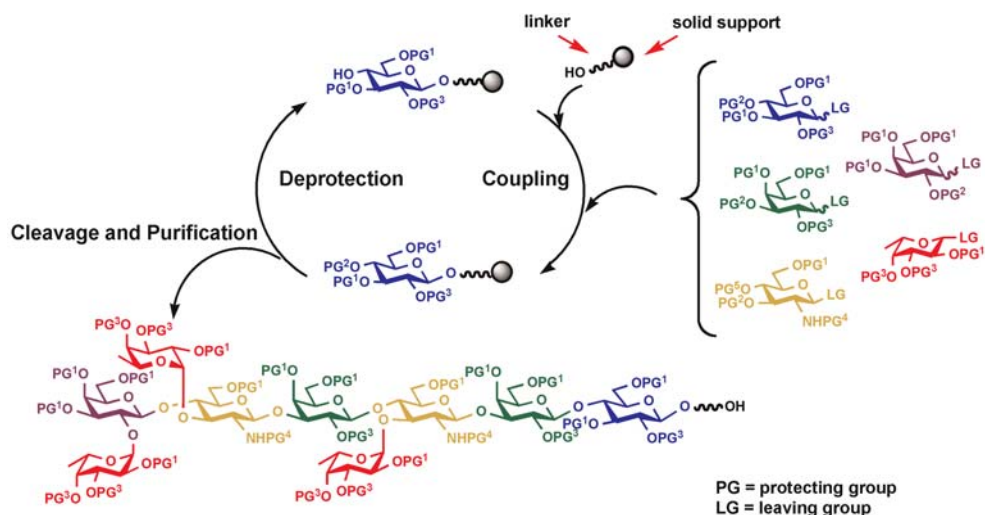
Enzymatic techniques rely on the high specificity of glycosyl transferase mediated glycosylations and are an alternative to traditional chemical synthesis [4]. Utilizing nucleotide diphosphosugars (NDPs) as building blocks, glycosyl transferases assemble complex carbohydrates in aqueous media. A major advantage of this method is the ability to prepare sophisticated structures without the need for protecting group manipulations on either the building blocks or the desired product. While certain carbohydrates can be prepared using a particular transferase, the narrow scope of transferase-mediated glycosylations necessitates the isolation and purification of multiple enzymes to synthesize diverse structures.

The desire to streamline the synthesis of carbohydrates and allow non-experts to prepare carbohydrates on demand has led to the design and evaluation of efficient one-pot methods. A solution-phase orthogonal method that relies on thioglycosides as glycosyl donors is the OptiMer™ strategy of Wong [5]. Analysis of the reactivity profiles for over a hundred different thioglycosides using a computer program allows for prediction of the optimal set of donors required to generate a given polysaccharide. The reactions are performed manually in solution with the oligosaccharide chain grown from the non-reducing to the reducing end.

Solution-phase oligosaccharide synthesis remains a slow process due to the need for iterative coupling and deprotection steps with purification at each step along the way. Solid phase synthesis has proven extremely efficient for the assembly of peptides and oligonucleotides as it does not require purification after each reaction step, utilizes excess reagent to drive reactions to completion and lends itself to automation. To alleviate the need for repetitive purification events required during solid-phase oligosaccharide assembly, solid-phase techniques have been developed [6]. Different approaches to solid-phase oligosaccharide synthesis had explored many critical aspects including the choice of synthetic strategy, differentially protected glycosylating agents, solid support materials, and linkers to attach the first monosaccharide to the support matrix.

We reported on the first automated solid phase oligosaccharide synthesizer in 2001 [7]. Attachment of the anomeric position of the reducing end sugar to the solid support allows for the step-wise incorporation of one mono- or disaccharide building block at a time. The use of UV active protective groups facilitates real time monitoring of the success of automated synthesis as is common for the synthesis of peptides and oligonucleotides. Glycosyl phosphates [8] and glycosyl trichloroacetimidates [9] proved to be useful building blocks for the automated assembly that also incorporated novel linkers [10] to connect the first sugar to the solid support. Based on innovative solutions to the protecting group, the building block, the linker and the analysis challenge, the first automated synthesizer was

based on a re-engineered peptide synthesizer [7]. Utilizing this automated synthesizer, a host of biologically important oligosaccharides was prepared to demonstrate the power of the approach. The synthesis of a complex carbohydrate like the Le^x-Le^y nonasaccharide antigen that is found on tumour cells was accomplished in less than one day when compared to well over one year using the most sophisticated solution phase synthesis methods [11].



Scheme 1. Automated solid-phase synthesis of a Le^y-Le^x nonasaccharide.

The currently available automated synthesizer has accelerated access to many carbohydrates several hundred-fold, still some sequences remain difficult to make and not all oligosaccharides can be assembled on solid support yet. The most time consuming step for the procurement of pure carbohydrates, is the synthesis of sufficient quantities of building blocks. Those building blocks will become commercially available in the near future and will greatly facilitate synthetic efforts. Improvements will include new building blocks and methodologies to access all possible linkages, accelerated protocols for the deprotection of synthetic oligosaccharides and better robotic systems that will facilitate access to multiple carbohydrates in parallel.

SCREENING INTERACTIONS INVOLVING CARBOHYDRATES

Glycoconjugates naturally decorate the surface of mammalian cells and are involved in cell-cell communication. Carbohydrate-protein interactions are common and carbohydrate-carbohydrate interactions have been implicated. Carbohydrates also have been found to specifically interact with bacterial RNA as is the basis for the mode of action for aminoglycoside antibiotics.

Carbohydrate-protein interactions

Recently, high-throughput screening methods to determine carbohydrate-protein interactions have been introduced rapidly [12]. Now, access to pure oligosaccharides is the limiting factor. Automated procurement of synthetic sugars has enabled the development of carbohydrate arrays. Many applications exist as the arrays can be used to discover novel carbohydrate protein interactions, define the epitopes recognized by disease-related or vaccine induced antibodies. Carbohydrate antigens for vaccine development can be identified using carbohydrate arrays. Initial carbohydrate microarrays in our laboratory focused on a panel of mannose containing oligosaccharides (Fig. 2a) [13]. The molecules were selected based on the glycans that decorate the viral surface envelope glycoproteins of HIV. The arrays were composed of a series of closely related structural determinants of (Man)₉(GlcNAc)₂. Using these arrays, precise profiles of the carbohydrate binding capacity of a series of gp120 binding proteins (DC-SIGN, 2G12, Cyanovirin-N and Scytovirin) were determined (Fig. 2b).

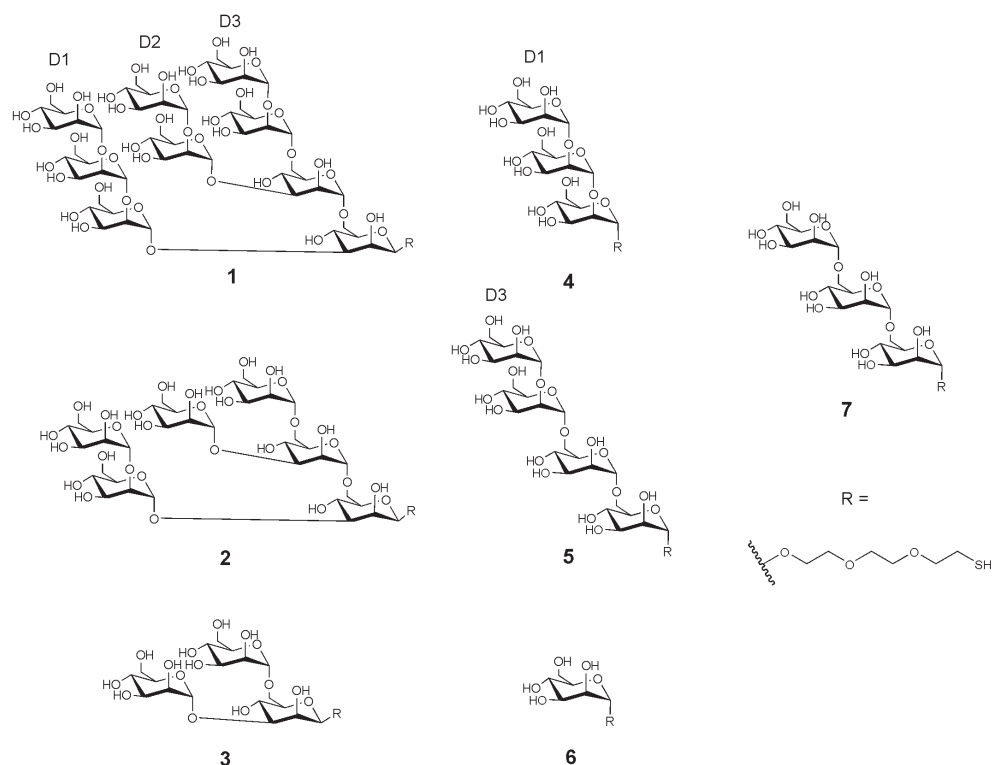


Figure 2a. Synthetic substructures of the triantennary *N*-linked mannose including a thiol-linker for immobilization. (Adopted from *Chem. Biol.* (2004) **11**: 875–881) [13].

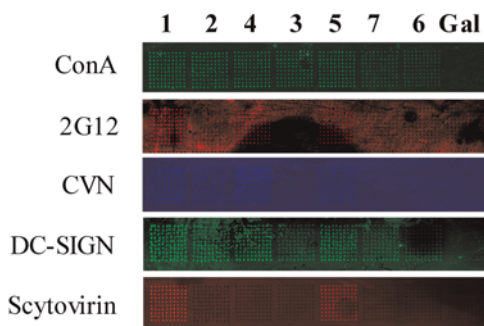


Figure 2b. Carbohydrate microarrays containing synthetic mannans **1** through **7** and galactose, printed at 2 mM. False colour image of incubations with fluorescently labelled ConA, 2G12, CVN, DC-SIGN and Scytovirin [13].

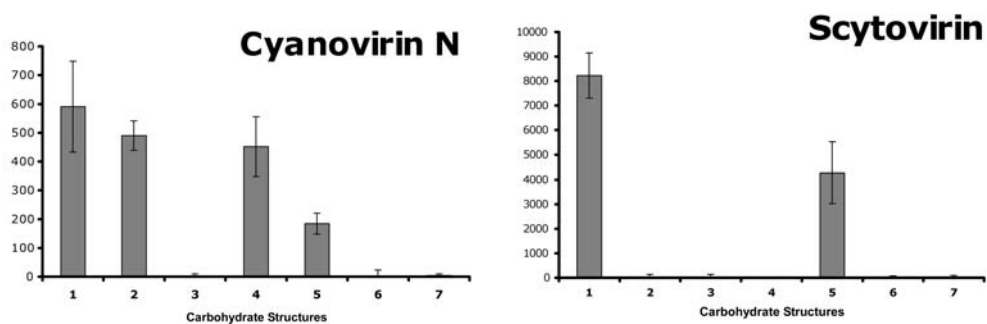


Figure 2c. Comparison of the binding profiles of fluorescently labelled Cyanovirin-N and Scytovirin, with mannans **1** through **7** [13].

The binding profiles of multiple proteins can be established by presenting various structural determinants of an important glycan on a single array, multiple proteins can be screened to determine their binding profiles. Figure 2b illustrates the carbohydrate of two potent HIV-inactivating proteins isolated from cyanobacterium, Cyanovirin-N (CVN) and Scytovirin [13]. The results illustrate that these two proteins recognize different structural motifs within the high-mannose series of structures arrayed. A single experiment yields significant data and saves much time compared with conventional methods.

Carbohydrate-RNA interactions

Aminoglycosides are carbohydrate antibiotics that contain amino sugars and are composed of two to five monomers. Clinically, these compounds are used as broad-spectrum antibiotics against a variety of important bacteria. The antibacterial effect is based on binding of the aminoglycosides to the bacterial ribosomes, thereby inhibiting protein synthesis. Therapeutic efficacy of aminoglycosides, however, has decreased recently due to increased

antibiotic resistance. In recent years, the incidence of resistant bacteria has increased. In order to combat the growing threat that bacteria pose to human safety, new antibiotics must be identified [14].

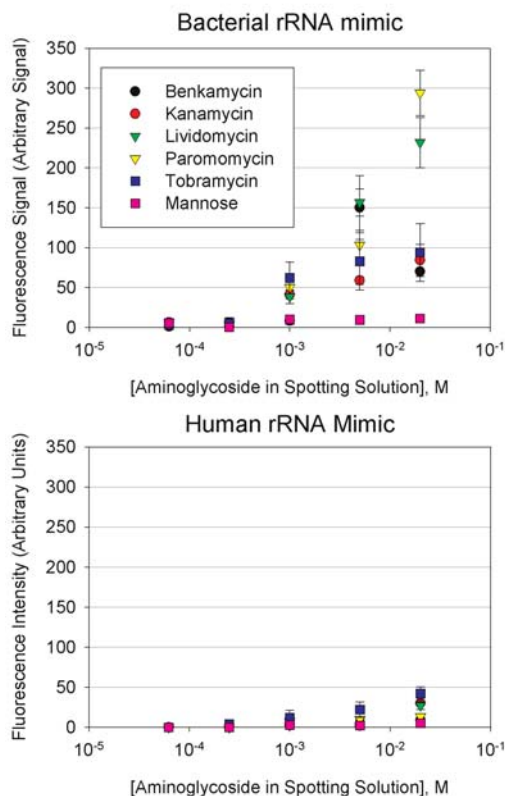


Figure 3. Fluorescence intensity of slides incubated with 10 μ M of human (18S) and bacterial (16S) hairpin mimics of the A-site in rRNA.

Aminoglycoside microarrays were constructed by non-specific immobilization of the antibiotics onto amine reactive glass slides using a DNA arraying robot [15]. This versatile platform was used to probe the interactions of aminoglycosides with a variety of targets. Arrays were probed with an RNA mimic of the bacterial and human A-sites (Fig. 3). Different RNA sequences were used to establish the microarray method to screen for RNA binding and binding specificity.

Carbohydrate arrays to detect bacteria [16]

Carbohydrate arrays can help to determine the carbohydrate binding specificity of intact bacterial cells. The carbohydrate-coated array surface presents carbohydrate ligands in a manner that facilitates multivalent binding. Cell adhesion on the arrays can be readily visualized using cell-permeable fluorescent dyes to stain the cells' nucleic acids. The array-based method enables assay miniaturization and requires only minimal amounts of ligand and cells when compared to solution measurements or experiments in 96-well plates. Carbohydrate-cell interactions can be detected in homogeneous and heterogeneous solutions that contain bacteria. Reliable detection even in complex mixtures that mimic body fluids illustrates the potential this method may hold in the future as a pathogen-specific diagnostic test.

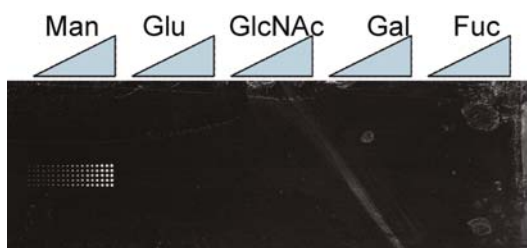


Figure 4. An image of a carbohydrate array after incubation with ORN178 cells that were stained with SYTO 83 cell-permeable nucleic acid staining dye. Each concentration was spotted with three rows of five spots. Each spot is the result of delivery of 1 nL of a 20 mM, 5 mM, 1.25 mM, 310 μ M, 63 μ M, or 15 μ M carbohydrate-containing solution. The spot diameter is \sim 200 μ m.

SYNTHETIC CARBOHYDRATE-BASED VACCINES

Vaccines are the most powerful and cost-efficient medical intervention in the control, prevention, and elimination of human infectious diseases [17]. Remarkable progress in the development of vaccines against many different human pathogens including polio, influenza, measles, diphtheria, tetanus, pertussis, varicella, mumps, rubella, hepatitis B, *Pneumococci*, and *Haemophilus influenza* type B, has been made [18]. Vaccination has enabled the eradication of smallpox and has decreased the incidence of once common childhood diseases.

Despite these successes, bacterial and viral infections in humans still represent major health problems, killing at least 15 million people annually. The search for new prophylactic and therapeutic vaccines to combat these infections has attracted considerable attention [19], albeit with only limited success. No effective vaccines against human parasites such as malaria, leishmaniasis, and schistosomiasis exist [20]. Vaccines such as the *Bacillus Calmette Guerin* (BCG) vaccine against tuberculosis, are often of limited efficacy. Thus, it is crucial to improve our understanding of the relevant glycan and protein antigens.

Vaccination is a way of inducing resistance to a foreign micro-organism by specially training the immune system. The body is exposed to innocuous biological material that mimics the infectious agent, but does not lead to infection or serious disease. The immune system is stimulated to generate antigen-specific antibodies and to neutralize the antigens. *Subunit vaccines* contain only parts of the micro-organism. Antigenic protein or carbohydrate fragments are either purified from natural sources or produced synthetically. Subunit vaccines against *Haemophilus influenzae* type B, Hepatitis A and B, diphtheria, and tetanus are currently on the market. The preparation of these types of vaccine has become possible due to improved synthetic methodology, improved purification protocols, advances in analytical methods, and an improved molecular and biochemical understanding of the pathogens. Advantages of fully or semi-synthetic vaccines include homogeneous and defined composition, easy modification to produce more potent analogues, less or no toxic side effects, and extended shelf-lives. Conjugate vaccines are an important class of subunit vaccines that consist of carbohydrate antigens covalently linked to an immunogenic carrier protein. Preliminary immunological studies with conjugate vaccines have already shown promising results in the treatment of various diseases including cancer, HIV, leishmaniasis, and malaria.

The first synthetic conjugate polysaccharide vaccine against *Haemophilus influenzae* type B has recently been approved and is now part of Cuba's vaccination program [21]. Many other carbohydrate-based vaccine constructs are currently being developed and tested or are in preclinical or clinical development [22]. Progress has been made in the development of synthetic carbohydrate-based conjugate vaccines against cancer [23] and HIV [24] as well as in the area of parasitic infections [25]. Synthetic carbohydrates will soon be a versatile basis for novel vaccines.

A CARBOHYDRATE-BASED VACCINE CANDIDATE AGAINST MALARIA

Malaria is the most devastating tropical parasitic disease in the world with the global number of cases continuing to rise. Together with tuberculosis and AIDS, malaria represents one of the major public health problems in more than 90 countries, mainly in tropical and subtropical regions of the world. Approximately 40% of the world's population lives with the risk of contracting malaria. Each year, malaria infects 5–10% of humanity and causes more than 300 million clinical cases. Mortality due to malaria is estimated to be between one and two million deaths per year. More than 90% of malarial infections and deaths occur in sub-Saharan Africa, mostly affecting young children under the age of five. Malaria represents one major cause of death in children worldwide and kills one African child every 20 seconds.

Already in 1886, Golgi proposed the existence of a malarial toxin as the causative agent of malarial pathogenesis [26]. The structural assignment [27] of the toxin (**8**, Fig. 5) was finally possible by chemical oligosaccharide synthesis [28]. The malarial parasite *P. falciparum* expresses a large amount of glycosylphosphatidylinositols (GPIs) anchored to proteins on its cell surface.

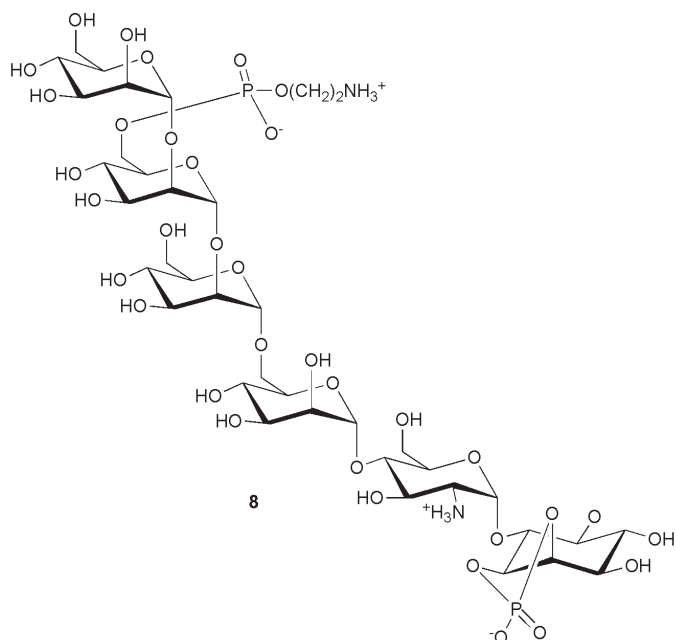


Figure 5. The GPI malaria toxin.

Malarial GPIs play a major role in the initiation and maintenance of the malarial inflammatory cascade. Initial studies, revealing that the malarial GPI can elicit an immune response in both rodents and humans, suggest that this compound has excellent properties for the development of an anti-toxin vaccine. Intense efforts are now underway to determine the minimal immunogenic structure and the structure-activity relationship by testing defined GPI structures in animals [29].

Due to the structural complexity of the GPI anchors, GPIs have been the target of intense studies. Various synthetic strategies have been developed [30]. The first chemical synthesis of the non-toxic malarial GPI glycan without lipid residues was accomplished using a linear solution-phase approach with six different building blocks. By combining automated solid-phase synthesis and solution-phase fragment coupling assembly was accelerated [31].

To prepare an immunogen, the synthetic pseudohexasaccharide was reacted with 2-iminothiolane to introduce a sulfhydryl linker at the ethanolamine unit. The resulting glycan was then conjugated to maleimide-activated keyhole limpet haemocyanin (KLH) as a carrier protein and this conjugate was used to immunize mice. Initial studies [28] showed that the synthetic malarial GPI conjugate was immunogenic in rodents, producing exactly the same immune response as the natural product. Immunized mice generated high titre immunoglobulin-g (IgG) to the synthetic GPI conjugate, whereas no reactivity was found in pre-immune sera or in animals receiving sham-conjugated KLH. The anti-glycan IgG antibodies cross-reacted with *P. falciparum* trophozoites and schizonts, as detected by immunofluorescence assay, but failed to bind to uninfected erythrocytes. In contrast to

malarial GPI, the core glycan of mammalian GPI is significantly modified [32], thus explaining the lack of reactivity. Antibodies from mice immunized with KLH-glycan were also able to neutralize the TNF- α level from macrophages induced by crude *Plasmodium* extracts.

The murine *Plasmodium berghei* ANKA malaria model is the best available model for certain aspects of lethal pathogenesis and corresponds well to several aspects of human severe and cerebral malarial syndromes. C57BL/6/J mice treated either with KLH-glycan or with KLH-cysteine in Freund's adjuvant were challenged with *P. berghei* ANKA. All infected sham-immunized and naïve control mice died within five to eight days with cerebral syndromes and exhibited severe neurological dysfunctions. In contrast, mice immunized with the synthetic KLH-glycan were significantly protected against severe malaria showing a reduced death rate. While between 58 and 75 % of the vaccinated mice survived until day 12, the survival rate for sham-immunized mice was only 0 to 9%. The level of parasites, however, does not differ significantly in immunized and control mice, indicating that prevention of fatality occurs without causing death of the parasites. Synthetic, non-toxic GPIs conjugated to a protein serve as anti-toxin vaccine candidates against malaria providing significant protection against malarial fatalities and pathogenesis.

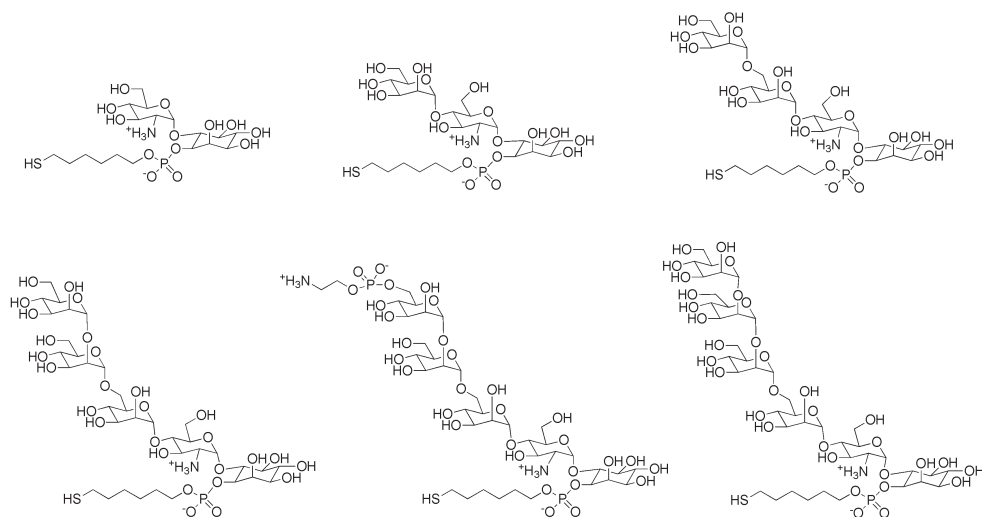


Figure 6. GPI oligosaccharides for microarray and affinity column experiments.

The preparation of a variety of different GPI oligosaccharides (Fig. 6) that differ in the number of mannose units has been accomplished recently [33]. These compounds currently serve as molecular tools for the examination of the biosynthesis, antigenicity, and serology of GPIs. The direct incorporation of a thiol group into the inositol moiety allows for the rapid conjugation of the glycans to carrier proteins and for convenient screening on carbohydrate chips [34]. The synthetic molecules are useful candidates to investigate the substrate specificity of GPI biosynthetic enzymes. They will be used as anti-toxin vaccine

candidates and will serve to reveal the minimal structural requirements necessary to elicit a protective immune response. Furthermore, they will find use as molecular probes to map epitopes of human anti-malarial antibodies and for other biological studies and will provide the basis for first detailed structure/activity relationship studies between GPI toxins and anti-malarial antibodies.

In summary, a variety of efficient synthetic methods for the preparation of malarial GPI glycans have been developed recently. Using these strategies, sufficient quantities of pure oligosaccharides can now be produced to support biochemical, biological, immunological and medicinal investigations. A better understanding of malarial pathogenesis will eventually help to discover an effective anti-toxin vaccine.

CARBOHYDRATE-BASED VACCINE CANDIDATE AGAINST LEISHMANIASIS

Leishmaniasis, another widespread tropical disease, is currently endemic in 88 countries on four continents. About 350 million people live at risk of leishmaniasis with a worldwide prevalence of 12 million clinical cases annually. The impact of leishmaniasis on public health has been greatly underestimated so far. It is estimated that about 1.5 to 2 million new infections and about 60,000 deaths occur each year [35].

Leishmaniasis is caused by several different species of protozoan parasites including *Leishmania donovani*, *L. tropica*, *L. infantum*, *L. major*, and *L. mexicana* and is transmitted by the bite of an infected female *phlebotomine* sand fly. Various forms of human leishmaniasis with a wide range of clinical symptoms and devastating consequences exist: *Visceral leishmaniasis* (kala azar) is the most lethal and severe form of leishmaniasis. The disease is characterized by regular bouts of fever, substantial loss of weight, anaemia, swelling of the spleen and the liver, and results in death, if untreated. *Mucocutaneous leishmaniasis* (espundia) produces lesions in the face that lead to partial or total destruction of mucous membranes of nose, mouth and throat cavities as well as of surrounding tissues. *Cutaneous leishmaniasis* is the most common form of the disease with 50–75% of the new cases, and is characterized by a large number of skin ulcers on the exposed part of the body. It causes serious disability leaving the patient permanently scarred. Leishmaniasis patients are usually treated with expensive antimony drugs, which require a lengthy therapy and show toxic side effects. Moreover, recently some *Leishmania* parasites have become resistant to some of the drugs. Several vaccine candidates are currently being explored including whole killed antigens as well as surface antigens; however, no effective vaccine has been developed to date.

The *Leishmania* parasites express large amounts of a complex oligosaccharide – a lipophosphoglycan (LPG) – on their cell surface. The LPG is composed of three parts, a GPI anchor, a repeating phosphorylated disaccharide fragment, and different cap oligosaccharides. Preliminary structure-activity relationship studies have been performed and suggest

that the cap glycan is used to attach the parasite to the digestive tract of the sand fly. Additionally, the cap is thought to contain the epitope responsible for recognition by the mammalian host macrophage.

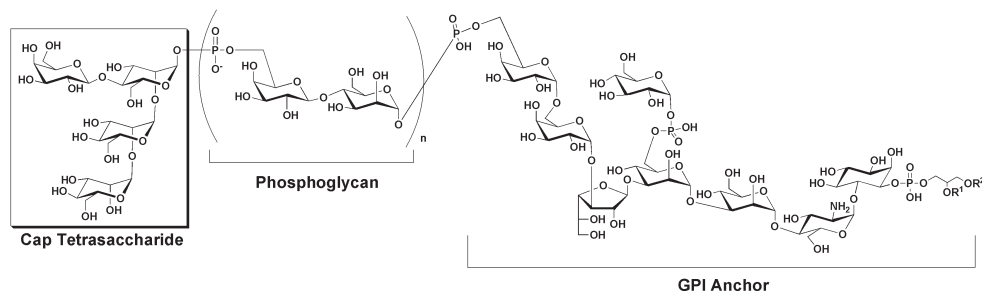


Figure 7. General structure of the leishmania lipophosphoglycan (LPG).

The branched tetrasaccharide cap that is found exclusively on the cell surface of *Leishmania* parasites, has been selected as the target for the development of an anti-leishmaniasis vaccine candidate. The automated solid-phase synthesis of the tetrasaccharide utilized the stepwise incorporation of three building blocks [36].

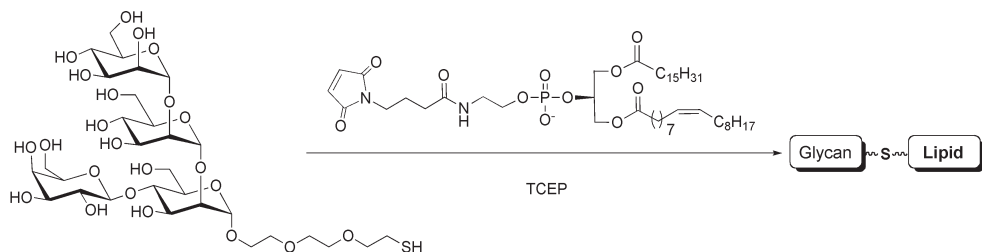


Figure 8. Cap tetrasaccharide glycolipid for virosome formation.

Novel virosomal formulations of the synthetic oligosaccharide were prepared and evaluated as vaccine candidates against leishmaniasis [37]. The lipophosphoglycan-related synthetic tetrasaccharide antigen was conjugated to a phospholipid and to the influenza virus coat protein haemagglutinin. These glycan conjugates were embedded into the lipid membrane of reconstituted influenza virus virosomes. The virosomal formulations elicited anti-glycan antibodies both of the IgM and the IgG class in mice. The antisera cross-reacted *in vitro* with the corresponding natural carbohydrate antigens expressed by leishmania cells. Further studies currently evaluate carbohydrate vaccine candidates for protection of dogs from leishmaniasis.

CONCLUSIONS AND OUTLOOK

The fundamental contributions of complex oligosaccharides and glycoconjugates to important biological processes are now understood in significant molecular detail. Specific types of carbohydrates are expressed on the cell surface of micro-organisms, and can be used as the basis for therapeutics, pharmaceuticals, diagnostics and vaccines. Glycans are often dominant antigens, and carbohydrate-based vaccine constructs have already been shown successfully to protect humans against various diseases. The isolation, purification, and identification as well as the preparation of oligosaccharides represented a major challenge to biologists and chemists. Recent advances in carbohydrate synthesis have led to innovative and efficient strategies facilitating the chemical and enzymatic preparation of diverse glycoconjugates. The introduction of an automated solid-phase synthesizer has greatly accelerated carbohydrate assembly. Linear and highly branched molecules are now accessible on this instrument, and its versatility has been demonstrated by preparing a series of biologically relevant oligosaccharides. These chemical approaches help to generate sufficient quantities of well-defined, homogeneous carbohydrate antigens for biological and immunological studies.

Parasitic infections, including malaria and leishmaniasis, are major public health problems, mainly afflicting humans in the developing world. Vaccines are the most effective and economical tool for the eradication of devastating infectious diseases, thus improving the quality of life. The role of vaccines will become increasingly important in the future to target infectious diseases, immunotherapy of tumours, chronic infections, autoimmunity and allergies. Vaccine candidates against malaria and leishmaniasis are currently being investigated. Advances in organic chemistry have led to completely synthetic carbohydrate-based antigens that have been used for the preparation of vaccine conjugates. Both malaria vaccine candidates are currently progressing through preclinical studies toward clinical evaluation.

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