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Review Article

Glycan synthesis: An update

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Abstract

Carbohydrate synthesis presents several challenges due to the intricate nature of molecules and their complex structures. Since certain pathogens are difficult to culture in the laboratory under some circumstances, and the isolation of glycans in pure structural forms is an arduous undertaking, the medicinal chemistry approach offers a fascinating and attractive option. The efforts being made to develop an automated system for oligosaccharide synthesis are expected to produce immense effects and revolutionize the understanding of the role of carbohydrates in biological systems. Glycovaccines aid in saving millions of lives around the world annually by protecting children and adults from bacterial infections. These vaccines further expand the therapeutic armamentarium against several microbial infectious diseases. This review highlights the different clinical applications of glycan and the progress made in their synthetic process.

Keywords: *Carbohydrates, Glycoproteins, Chemoenzymatic synthesis, One-pot synthesis, Glycosyltransferase, Glycosidase, Glycovaccines*

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INTRODUCTION

Glycans and glycosylated biomacromolecules (glycoproteins and glycolipids) perform essential functions in biological and pathophysiological processes. Studies have elucidated the fundamental roles of glycoproteins in several processes such as cancer metastasis, neuronal development, hormone activities, infectious diseases, and immune responses [1]. The glycans expressed on the surfaces of all host cells which produce a layer known as the glycocalyx, are recognized, and utilized by pathogens for cell penetration and evasion of the immune system. Moreover, due to their crucial function in communication, an important feature of glycans is exhibited by their post-translational task in the quality control of polypeptide biosynthesis [2]. Several polypeptides are folded inappropriately due to inappropriate glycosylation process. It is believed that if biosynthesized polypeptides are not appropriately folded, the glycan moieties will be incorrectly positioned, thereby impeding the trimming steps that lead to expulsion of the polypeptide via the endoplasmic reticulum-associated protein degradation route. In addition, glycans appear to be essential in the stabilization of the tertiary structure of proteins and facilitating their folding by protecting proteins from proteolysis [3].

The transversal role of glycans in different biological processes may be elucidated through their variegated range of structures. With respect to the polymerization processes, proteins and nucleic acids are linear in structure, and their restricted basic groups (20 amino acids for proteins and 4 nucleotides for DNA and RNA) limit the number of structural variations [4].

Thus, the classical views of carbohydrates as sources of energy for the living systems and as skeletal support have been modified. Currently, a wider spectrum of functions has been attributed to them, and several scientists have elucidated the correlation between glycans and various diseases which facilitated the discovery of novel therapeutic agents [5]. For this reason, in-depth investigations on the importance of glycans may help to identify new therapeutic strategies for illnesses that are difficult to cure, i.e., cancer and viral and bacterial infections. There is need to develop highly efficient, economical, and straightforward synthetic or chemo-enzymatic methods so as to achieve robust progress in these therapeutic strategies. However, this may not be easily achievable, especially in the synthesis of glycans, as will be explained downstream in this review article.

This review was aimed at providing an overview of recent strategies and concepts which make the synthesis of glycans a feasible undertaking. It is hoped that this will contribute to enhancing success in the development of novel therapeutic glycol medicines that target glycans.

Therapeutic potential

Diagnosing several diseases through chemical characterization of alterations in oligosaccharides in blood and/or urine is related to the unusual distribution of cell surface-expressed or secreted glycomes [6]. In addition, the stage at which disease manifests may be recognized by glycosylated variants of proteins known as glycoforms [7]. Therefore, studies on this aberrant glycosylation by glycobiologists may reveal extremely important information on their potential as invaluable diagnostic tools.

In addition, the immune system utilizes glycans as biomarkers for distinguishing its cells from foreign antigens (human or microbial antigens) [8]. For example, some microorganisms such as bacteria, viruses, and parasites that express glycans on their surfaces use them to initiate the infectious process through interaction with the host cell lectins (i.e., glycan-binding proteins) [9]. Moreover, glycans may be used by invaders as a trojan horse strategy to deceive the immune system.

Carbohydrate-containing drugs

The clinical applicability of glycobiology is evident in the several numbers of commercially available, glycan structure-based drugs such as anti-thrombotic heparin [10], anti-influenza virus drugs zanamivir and oseltamivir [11], and anti-diabetic drugs voglibose, miglitol and acarbose) [12]. Others are aminoglycoside antibiotics [43] [13], and nucleoside inhibitors (anti-HIV and anti-herpes) [14]. Indeed, molnupiravir (approved and launched in the United Kingdom in 2021) became the first oral antiviral drug for the management of COVID-19 [15]. These drugs provide the basis for glycan-based drug discovery (Figure 1).

It is clear that the road to comprehending the role of glycans is long, but so much progress has been made, and more will have to be realized in the field of glycobiology, in order to pave way for the development of new therapeutic applications, especially if access to large scale synthesis of glycans is achieved.

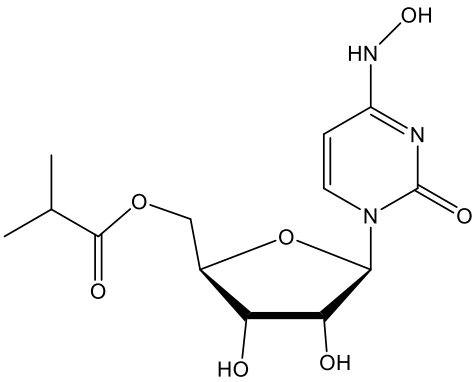


Figure 1: Chemical structure of molnupiravir

Neoglycoprotein-based vaccines

Synthesized glycans or their glycoconjugate derivatives may permit the preparation of novel set of vaccines against bacteria [16], viruses [17], and cancer cells [18]. However, vaccines which are composed exclusively of glycan moieties usually are thymus-independent antigens which stimulate weak immunity, specifically in newborns [19]. The most important inconvenience is that polysaccharides are T-cell-independent antigens and do not efficiently elicit T-helper-dependent stimulation and class triggering of B-cell-mediated immune response [20]. Carbohydrate-conjugate vaccines such as neoglycoprotein vaccines made from oligo- or polysaccharides linked to immunogenic carrier

proteins, are strongly effective and specific, and they create long-lasting immune responses with the help of B- and T-cells [21].

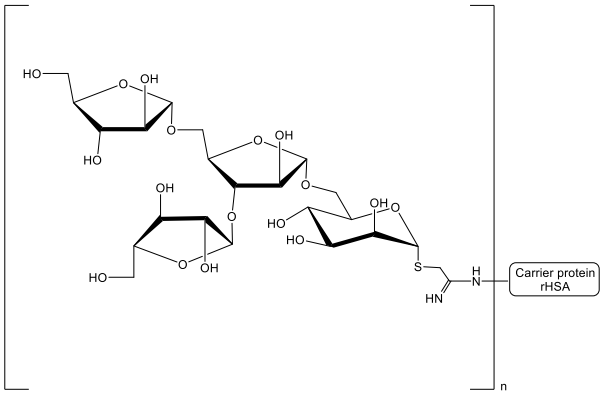


Figure 2: AM analogue as a promising neoglycoprotein candidate vaccine

Tuberculosis vaccines

Approximately 25 % of the world's population is infected by tuberculosis (TB)-causing *Mycobacterium tuberculosis.* This is expected to result in 1.65 million deaths annually, which ranks TB among the top 10 causes of mortality in the world [22]. Despite the availability of anti-TB drugs, there has been a rapid increase in multiple-drug-resistant (MDR) and extensive-drug-resistant (XRD) bacteria in recent years, resulting in limited drug effectiveness and aggravation of TV crisis [23]. Live attenuated *Bacillus* Calmette-Guérin (BCG) vaccine is the only existing TB vaccine [24]. However, it does not provide any meaningful protection against the most widespread and transmissible form of adult pulmonary TB [25]. Moreover, it is only effective in children [26]. The incidence of TB in adults has led to intensified awareness of the synthesis of saccharide antigens for the development of novel neoglycoprotein-based vaccines for TB prevention. In this case, the choice of potential antigen is based on analysis of the complex glycolipid components of the cell wall structure of *M. tuberculosis*. Owing to their biological importance, extensive attempts have been made to synthesize *M. tuberculosis* capsular oligosaccharides [27]. A great deal of this effort has centered on the synthesis of the non-reducing moiety of the lipoarabinomannan (LAM). In 2020, Zhihao *et al*. used glycol-conjugation of immunogenic carrier proteins rHSA with 2-iminomethoxyethyl (IME) thioglycoside linker at the reducing terminal of the oligosaccharide, resulting in the production of neoglycoproteins. The strongest response was observed using biological evaluation through *ex-vivo* tests with a tetrasaccharide synthesized from arabino-mannan (AM) motif-containing D-arabinose units and mannose, with branched points at α-(1→5) and α-(1→3) (Figure 2) [28].

Hemophilus influenza Type B vaccines

Almost all commercially available glycoconjugate vaccines are based on glycans purified from natural sources [29]. The only exception is Quimi-Hib, a Cuban semi-synthetic Hib vaccine approved for the elimination of juvenile meningitis. This emphasizes the potential of neoglycoprotein-based vaccines. Encouraged by the accomplishment in vaccines, e.g., purified Hib capsular polysaccharides, totally synthetic portions of the native polysaccharides were coupled to a carrier protein (Figure 3) [30]. The immunogenicity of the semi-synthetic conjugate was similar to that of the native polysaccharide conjugated to the same carrier protein. Thus, it is considered the first effective synthetic, large-scale polysaccharide antigen produced for vaccine manufacturing. This glycoconjugate vaccine (Quimi-Hib) which was authorized and marketed in Cuba in 2003, is marketed in several South American countries, and it has been regularly utilized for preventive immunization of infants and children [31].

The handling of synthetic antigens, particularly glycan conjugates in which the immunizing agent is synthesized chemically with atomic level accuracy, provides important potential for further developments alongside this line. This is predominately due to the controlled production of a homogenous composite that minimizes batch-to-batch irregularity, thereby increasing quality control standards. This might result in lower production costs when compared with classical vaccines.

Cancer immunotherapy

Since glycobiology has been recognized as a fundamental area in cancer studies, cancer immunotherapy has ultimately attracted considerable awareness due to its potential to serve as a highly potent and promising cancer treatment and prevention [32]. Immunotherapy is aimed at stimulating patient's immune defense so as to eliminate cancerous cells utilizing monoclonal antibodies (mAbs). These mAbs could be specifically linked to cancer cells or a vaccine that elicits a specific immune response to cancerous cells in order to arrest the migration and invasion of tumor cells from their original site [33].

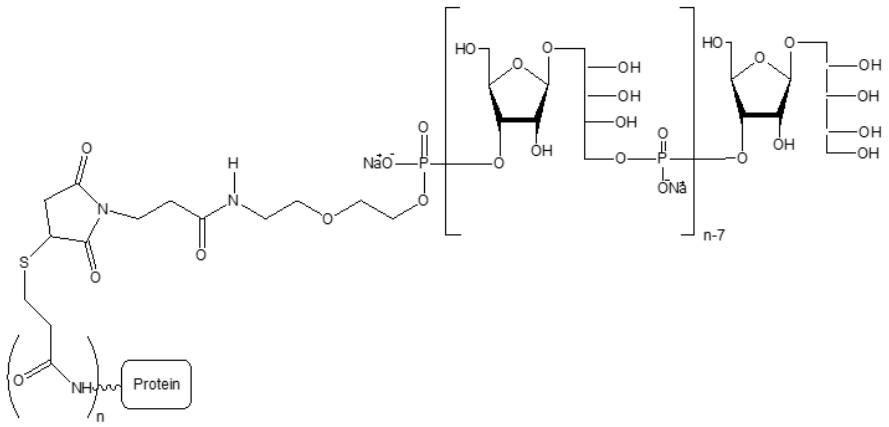


Figure 3: *Haemophilus influenzae* Type B glycoconjugate vaccine (Quimi-Hib vaccine)

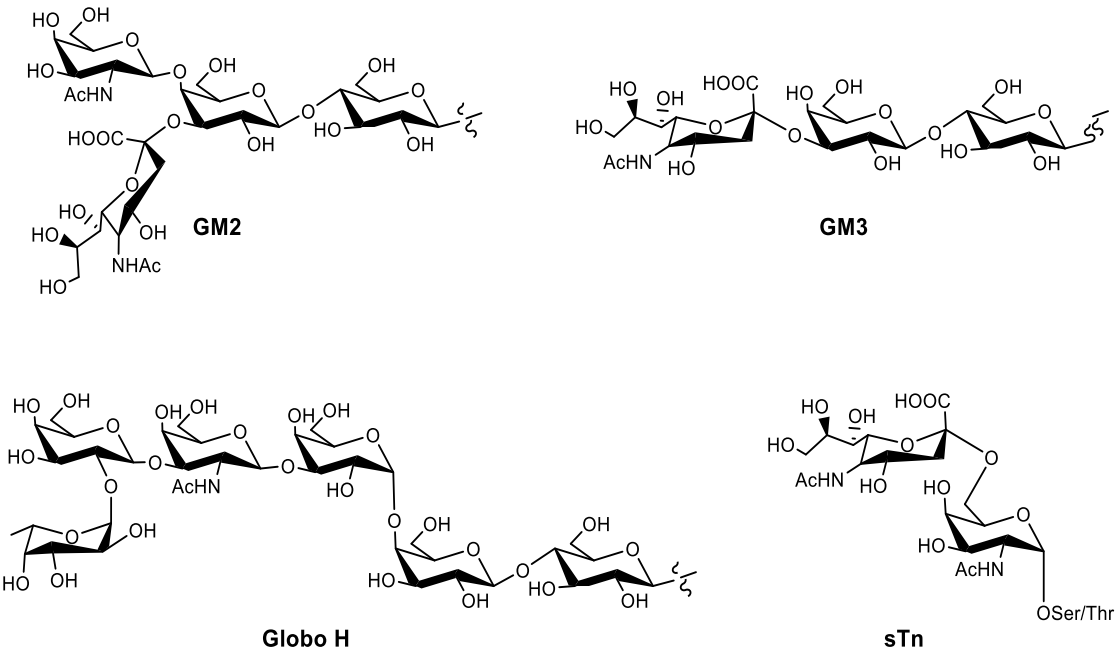


Figure 4: Structures of tumor-associated carbohydrate antigens (TACAs)

As a result of this, it is supposed that glycans distinctively or abnormally expressed on the surface of tumor cells, known as tumor-associated carbohydrate antigens (TACAs), are potential targets [34]. This is due to the abundance and exposure of the TACAs on the cancer cell surface and their involvement with different stages of cancer pathogenesis, including proliferation, invasion, angiogenesis, and metastasis [35] (Figure 4). Thus, TACAs may be useful in the development of novel cancer glycan-based therapeutic drugs [36].

Nevertheless, serious challenges are associated with TACAs because they are typically poorly immunogenic structures, and they induce T cell-independent immune response [37], whereas T cell-mediated immunity is crucial for cancer immunotherapy.

A further concern is that majority of TACAs are not recognized by the immune system [38]. Although the precise mechanism is not fully clear, a low level of expression of TACAs in healthy cells or at a specific development stage, and their structural similarities to normal antigens, may at least be partially responsible for immune tolerance [39]. In fact, instead of being tumor-specific, most TACAs are either excessively expressed 'self' glycans or their biosynthetic intermediates. Thus, it is difficult to create effective vaccines from TACAs. However, it has been established that covalent coupling of carbohydrates to immunologically active proteins remarkably improves their immunogenicity and converts them from T cell-independent antigens to T cell-dependent ones.

Livingston and co-workers have elucidated that KLH provides T cell epitope to TACAs [40]. It is also reported in literature that the linker utilized to conjugate glycans and proteins may exert an important effect on the immunological features of these resultant conjugates. For example, some linkers may elicit antibodies, but others may suppress the immune response to the target antigen [41]. Consequently, it is critical to utilize immunologically inactive linkers to produce TACA-based cancer vaccines [42]. For example, the KLH conjugates of GM2 [43] and sTn-KLH conjugate [96], have all been tested in Phases II and III clinical trials, respectively [44].

Unfortunately, up till now, due to drawbacks related to efficacy and toxicity, there is no TACA-based cancer vaccine approved for clinical use. Despite this, the development of future carbohydrate-based vaccines that hopefully will lead to successful clinical applications remains a very active and attractive field. To accomplish this, a robust synthetic glycan method must be developed, due to the existence of glycoforms expressed on the cell surfaces.

Carbohydrate assembly

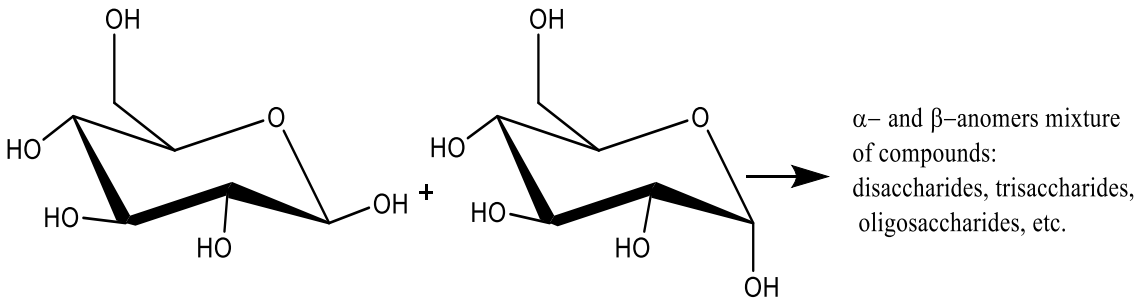
Chemical synthesis

Obtaining pure carbohydrates of well-defined structures is a difficult task because, among all biopolymers, carbohydrates are the most perplexing, and they are indispensable study tools in glycobiology. Unlike proteins and nucleic acids which can be obtained in pure homogeneous structures utilizing biotechnological approaches such as recombinant DNA and polymerase chain reaction (PCR) [45], carbohydrates biosynthesized in living systems are heterogeneous. Furthermore, carbohydrates are more complex in structure because the glycosidic bond between monosaccharides occurs with alpha or beta linkages. The possibility of bonds with different hydroxyl groups at different positions of the monosaccharides gives glycans a high degree of complexity. In addition, the quantities that can be purified in pure structures from biological systems are relatively modest, and not every bacterial strain is easily cultured [46]. In carbohydrate chemistry, chemical synthesis is utilized to obtain homogeneous glycans in a greater quantity than are obtainable from most cellular production systems, but this type of production usually results in small amounts of impurities [40]. Biocatalysts may be utilized in combination with chemical synthetic methods to make the glycan synthesis process more feasible and simpler. Moreover, obtaining well-defined glycan structures will enhance the feasibility of structure-activity relationships (SARs) studies [47]. Additionally, it will be possible to employ chemical synthesis to incorporate glycans into homogenous neoglycoproteins [48].

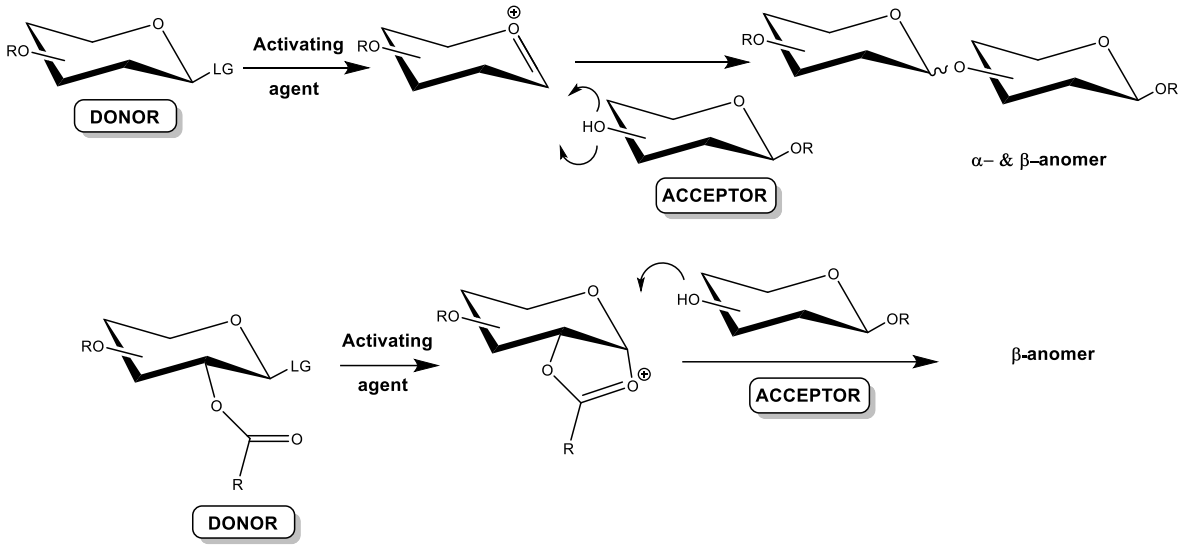
Problems associated with oligosaccharide assembly

Various monosaccharide building blocks are produced in nature and are thus commercially available in their pure forms. Many methods of oligosaccharide syntheses involve the joining together of these monosaccharide units to build larger structures [49]. Several complicated factors must be associated with this simple process. For example, the preparation of disaccharides between two unprotected monosaccharides results in a mixture of different isomers, as shown in Scheme 1.

The chemical synthesis of polysaccharides is a difficult undertaking which entails the application of complex manipulations involving many protective/de-protective steps due to presence of different hydroxyl groups which possess similar reactivity except the anomeric hydroxyl. Carbohydrate synthesis usually involves the preparation of two building blocks, i.e., glycosyl donors and glycosyl acceptors. The glycosyl donor bears a good leaving group, and the glycosyl acceptor bears only one free hydroxyl group.



Scheme 1: Synthesis of disaccharides between two unprotected monosaccharides



Scheme 2: Stereochemical issues in the synthesis of carbohydrates

For example, the synthesis of disaccharide with a defined isomer requires an acceptor bearing only one hydroxyl group at the desired position, and a donor presenting a good leaving group that will react with the free acceptor hydroxyl group. This facilitates regioselective addition to obtain the desired disaccharide [50].

Therefore, the first challenge to face is the hydroxyl protective groups that have to be selectively added and then removed from carbohydrate structure, choosing appropriate groups a priori fundamental. Compared to the chemical approach, enzymatic biocatalytic deprotection selectively exposes one hydroxyl group to the regioselective coupling of another monosaccharide unit [51]. Furthermore, the second challenge to face is the stereochemistry of the glycosidic bond formed through the activation of a glycosyl donor to generate a highly reactive electrophilic species that conjugates with the glycosyl acceptor hydroxyl group, thereby creating a glycosidic linkage. This coupling reaction results in the formation of α- and β-glycosidic linkages. For this reason, it is fundamental to generate stereospecific glycosidic linkages. A recent method utilized to control the stereochemistry at the anomeric carbon requires the use of certain protective groups such as ester or amide moieties, on the 2-hydroxyl group (Scheme 2). Under glycosylation reaction conditions, these "neighboring protecting groups" produce cyclic oxonium ion intermediates that protect one face of the monosaccharide, leading exclusively to the formation of a *trans* glycosidic linkage. The opposite anomeric stereochemistry, which is called a *cis* glycosidic bond, is more challenging to make with high specificity because "neighboring group participation" is not feasible [52].

A technique that may facilitate and improve the synthesis of carbohydrates comes from orthogonal protecting group strategies used over the last decades, which involve addition and removal of groups under specific reaction conditions with the aim of leaving the remaining protecting groups intact [53]. The literature contains descriptions of several orthogonal protecting groups such as halobenzyl ether [54], 2- (allyloxy)phenylacetyl ester (APAC) [55], Fmoc [56] and diethylisopropysilyl (DEIPS) [57], as well as the conditions used for their chemo-selective de-protection. However, Hahm *et al* described automated synthesis of oligosaccharides containing multiple *cis*-glycosidic linkages fascinatingly, utilizing monosaccharides equipped with remote participating protecting groups [58].

However, the chemical synthesis of highly pure glycans is a great task due to their structural complexities. These polysaccharides are synthesized through traditional chemical procedures requiring several protection/ deprotection reactions that negatively influence the total yield, reaction time and process expenses.

Chemoenzymatic synthesis

This approach is relatively new. This procedure is characterized by the usage of one protective group (acetyl ester) through all the planned synthetical steps. It represents a simple, effective, and environmentally friendly alternative to the conventional synthetic methods in

carbohydrate chemistry which involve the use of hazardous reagents and management of various protective groups [59].

Hydrolases: regioselective de-protection of protected sugars

Lipases or triacylglycerol acylhydrolases are hydrolytic enzymes that catalyze the reversible cleavage of ester bonds in triglycerides [60]. They are the most widely used enzymes in biotransformation since they are stable and easy to handle, and they have a rather broad substrate specificity and high enantioselectivity and regioselectivity. Lipases represent an alternative to the chemical methods for the regioselective deprotection of carbohydrates [61,62].

The classical approach for the synthesis of glycans on the orthogonal protective route is extremely complex and characterized by low controls of the reaction regiospecificity and stereospecificity [63]. An alternative strategy entails using acetyl ester as the sole protective group for all the hydroxyl groups present in glycan. Subsequently, mono-deprotected sugar building blocks are prepared through enzymatic regioselective deprotection with immobilized hydrolases. This approach allows for the synthesis of complex oligosaccharides, thereby overcoming the complexities of the classical orthogonal protection strategy [53]. The first chemical and enzymatic attempt to hydrolyze only one specific position on monosaccharides afforded a unique deprotection in the anomeric position (C-1), as shown in Scheme 3 [64].

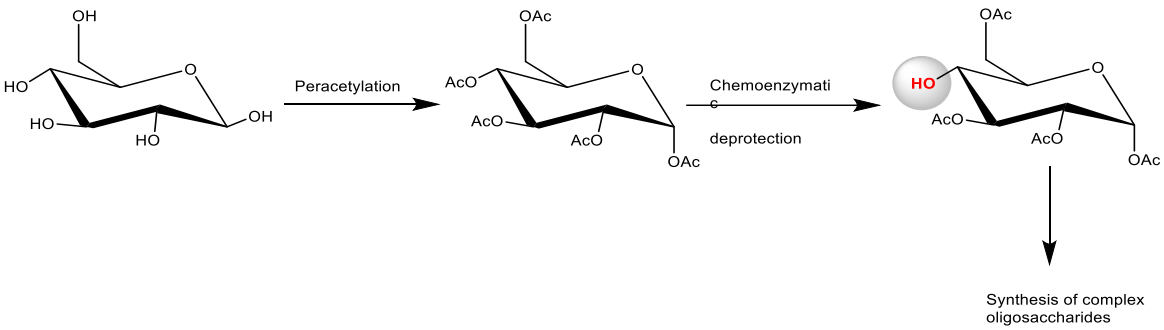
Enzymatic synthesis

To deal with problems such as regioselectivity and stereospecificity associated with glycan biosynthesis, living systems have developed enzymes that couple monosaccharides efficiently. On the other hand, chemical synthesis permits the preparation of various natural and unnatural structures, but it requires the use of large number of protective groups and the preparation of specialized precursor compounds [65]. In contrast, enzymes (biocatalysts) such as glycosyltransferases and glycosidases, are typically much less flexible and available but do not require protecting groups or elaborate precursors, and they produce glycosidic linkages with perfect regiochemical and stereochemical controls, and under mild reaction conditions [66]. However, the strategy of utilizing enzymes has its challenges. For example, the relatively high cost of certain enzymes, as well as solubility problems, as some substrates require solubilization in organic solvents. These solvents are not conducive environments for enzymes because enzymes lose activity in organic solvents due to denaturation.

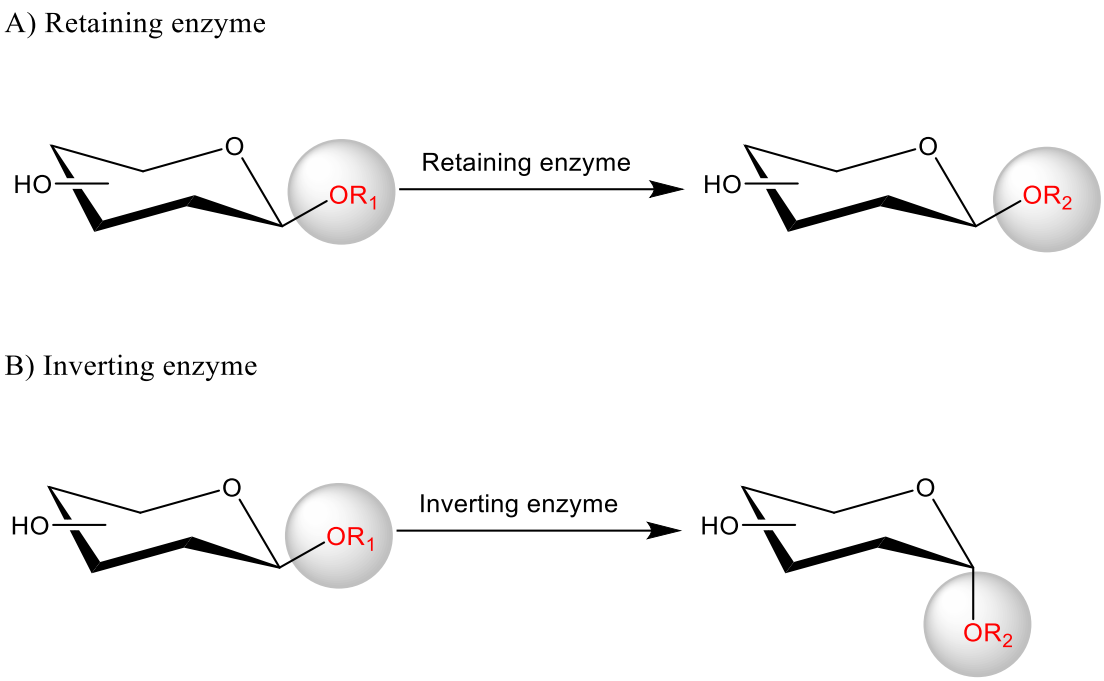
Glycosyltransferases and glycosidases

In cell biology, glycosyltransferases (glycan-adding enzymes) catalyze the biosynthesis of glycosidic structures through the transfer of activated glycosyl-nucleotide donors to acceptors such as sugars, proteins, lipids and DNA. The potential advantages of glycan synthesis using glycosyltransferases are immediately obvious: the reactions result in high yields, perfect regiospecific and stereospecific glycosidic bond formation, and they are environmentally friendly [67].

However, utilizing glycosyltransferases on a large scale has several significant limitations, including low catalytic activity, sensitivity to environmental conditions, the necessity to use expensive cofactors (UDP-GlcNAc), less clear mechanism of glycoside bond formation, and the potential for enzyme inhibition or denaturation under certain reaction conditions [68].



Scheme 3: Acetyl ester is the sole protective group in the chemoenzymatic strategy



Scheme 4: Mechanistic classification of α/β-glycosynthases. A: retaining enzyme; B: inverting enzyme

In contrast, glycosidases (also called glycoside hydrolases, glycan-removing) catalyze the hydrolysis of glycosidic bonds and usually split glycans into smaller units or monosaccharides. To use glycosynthases for the synthesis of oligosaccharides, the normal function of a class of glycosidases (recently developed using genetic engineering) must be reversed by replacement of the active site nucleophilic group with a non-nucleophilic residue [69]. However, in the presence of a glycosyl donor with a good leaving group, the mutant enzyme catalyzes the transglycosylation to an acceptor, with an excellent yield. In this reverse reaction, the glycosynthases catalyze the formation of a glycosidic bond between the glycosyl donor and an acceptor molecule such as a protein or another sugar molecule. This reverse reaction is used to synthesize specific oligosaccharides with defined structures and sequences. In the literature, there are two types of glycosynthases, based on reaction mechanism: *inverting* and *retaining* α/β-glycosynthases, as shown in Scheme 4 [70].

In general, glycosidases are cheap biocatalysts that are more resistant to protein denaturation and more readily available than glycosyltransferases, and they are also more tolerant to variations in substrate structure. Glycosidases are produced by a wide range of microorganisms, and they are readily purified from natural sources or produced recombinantly in large quantities. In contrast, glycosyltransferases are often more difficult to produce and purify due to their complex structures and specific substrate requirements [71].

CONCLUDING REMARKS

The development of innovative glycan synthesis approaches is a key driver in advancing glycan-based drug discovery. These approaches enable scientists to create a wide range of glycan structures, study their interactions with biological systems, investigate their structure-activity relationships and design novel therapeutic interventions that harness the unique properties of glycans. The state-of-the-art progress that is taking place in the synthesis of carbohydrates is expected to lead to the downstream development of a more rational method for the design of vaccine antigens rather than the existing trial-and-error approach, and it has the potential to simplify the procedure of quality control. Regioselective protection and stereoselective glycosylation are usually required. Synthetic studies and investigations on complex glycans help to get large-scale homogeneous stereo-defined structures which are useful for glycobiology studies.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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