Carbohydrates

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Toward Automated Oligosaccharide Synthesis

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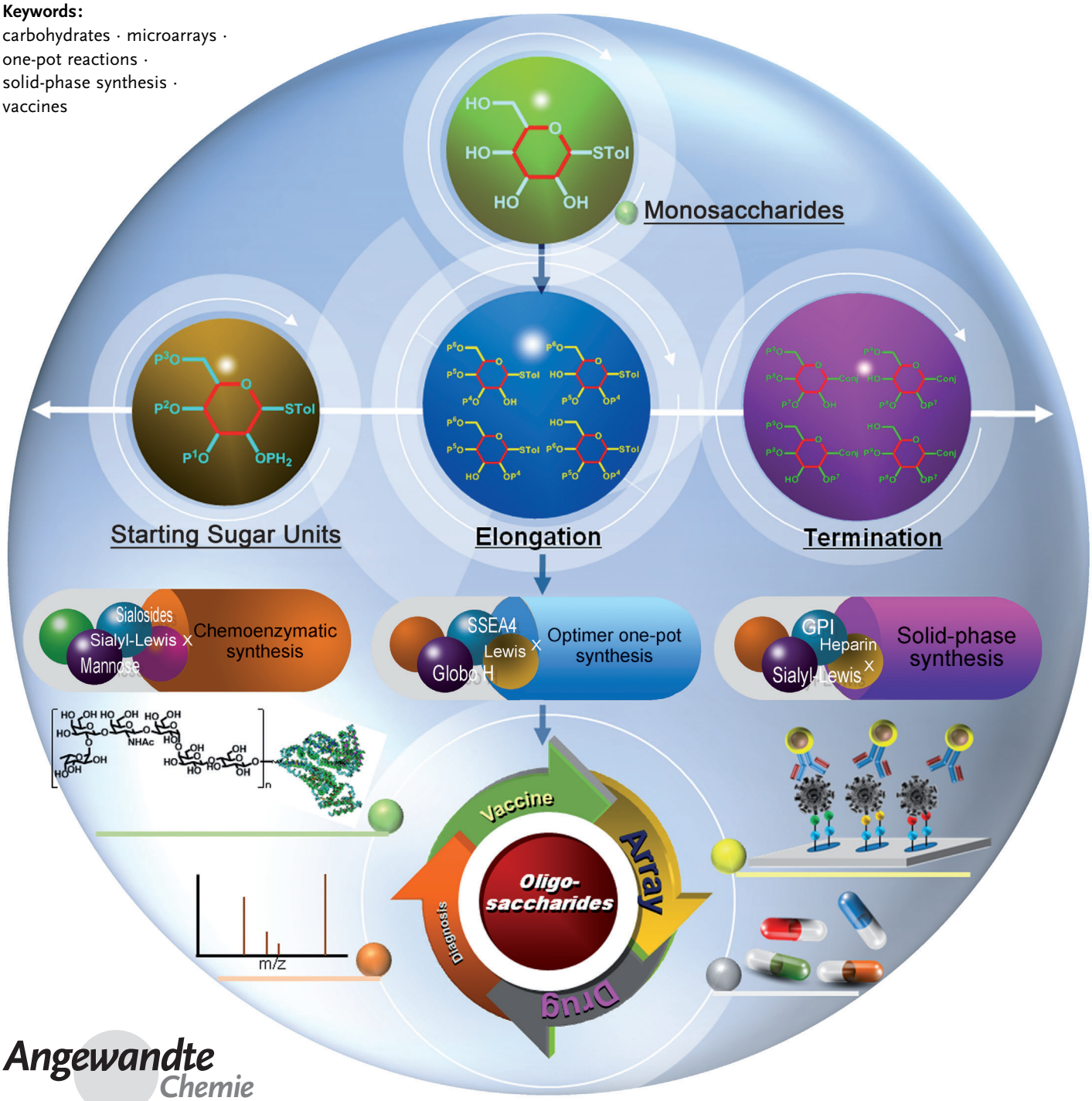

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Carbohydrates have been shown to play important roles in biological processes. The pace of development in carbohydrate research is, however, relatively slow due to the problems associated with the complexity of carbohydrate structures and the lack of general synthetic methods and tools available for the study of this class of biomolecules. Recent advances in synthesis have demonstrated that many of these problems can be circumvented. In this Review, we describe the methods developed to tackle the problems of carbohydrate-mediated biological processes, with particular focus on the issue related to the development of the automated synthesis of oligosaccharides. Further applications of carbohydrate microarrays and vaccines to human diseases are also highlighted.

From the Contents

1. Introduction

Beyond their traditionally accepted roles as energy sources and structural polymers, it is now well established that carbohydrates, one of the three major classes of biopolymers, are crucial in numerous biological processes, including viral and bacterial infection, angiogenesis and tumor cell metastasis, toxin interaction, inflammation and immune response, cell growth and proliferation, and many other cell–cell communications.[1] Compared to proteins and nucleic acids, the naturally occurring oligo- and polysaccharides, glycoproteins, glycolipids, as well as glyco-containing antibiotics and natural products are structurally diverse and complex so as to allow the encoding of required information for specific molecular recognitions. The information embedded in these structures, particularly the posttranslational modification of proteins, has attracted great interest and fueled the emerging field of glycomics.[2] All these investigations of biological functions and structure–activity relationships (SARs) require homogeneous materials with well-defined carbohydrate structures. However, the complex carbohydrates, which commonly exist in microheterogeneous forms, cannot be obtained easily from natural sources in acceptable purity and quantity. Thus, significant efforts have been devoted to the development of chemical and enzymatic methods for the synthesis of structurally well-defined carbohydrates and conjugates.[3]

It can be seen from the methods available for the synthesis of the three major classes of biopolymers that the preparation of carbohydrates is definitely more difficult and complicated than that of peptides/proteins and nucleotides/DNA and RNA. Nucleic acids can be synthesized by chemical and biological methods with the aid of the polymerase chain reaction, and protein sequences, which are encoded by DNA, can be easily determined, produced, and manipulated through recombinant DNA technology. Since no regio- and stereochemical issues are involved in the sequential coupling steps for the construction of amide or phosphodiester bonds, automation of the synthesis is possible for these linear polymers (polynucleotides and polypeptides) by using single protecting-group strategy in iterative processes. Saccharides are, however, assembled through stereospecific linkage of monomers, often branched and made by using a diverse set of enzymes; therefore, there is no information carrier that encodes a particular saccharide sequence.[4] Two major challenges, namely the regioselective protection and deprotection of polyhydroxy groups as well as the stereoselective assembly of glycosidic linkages, are frequently encountered in carbohydrate synthesis, especially with an ultimate goal of developing an automated method. Although the stepwise solid-phase synthesis of oligosaccharides has been developed, the complexity of protecting-group manipulation still remains a major challenge. On the other hand, automated enzymatic oligosaccharide synthesis is limited to the availability, specificity, and stability of enzymes. Thus, a strategy combining

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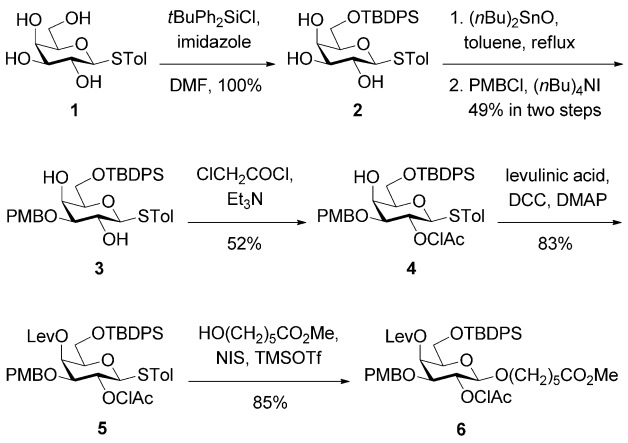
"regioselective one-pot protection" and "automated and programmable one-pot glycosylation" (Figure 1) has been developed to tackle some of these problems. In this Review, we emphasize recent progress in the regioselective protection of monosaccharides, automated and programmable chemical glycosylation, and enzyme-catalyzed glycosylation as a means for rapid access to complex oligosaccharides, and their applications to the development of microarrays and vaccines.

2. Regioselective Protection of Monosaccharides

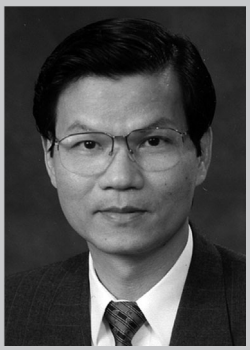
Designing protecting-group strategies is a crucial step in the initial stage of any sugar synthesis. A frequently encountered problem is the regioselective installation of an orthogonal set of protecting groups in the saccharide building blocks so as to have the broadest possible flexibility without affecting the remaining ones. Protecting groups may strongly influence: 1) the reactivity of a glycosyl donor/acceptor, 2) the stereoselectivity of glycosidic bond formation, and 3) the ease of final deprotection. Since several reports have already addressed these issues,[5] this Review focuses on the development of recent regioselective protection strategies.

2.1. Stepwise Protection Strategy

The differentiation of all the hydroxy groups on a sugar molecule is a challenging task. The design of monosaccharide building blocks by using an efficient orthogonal protection/deprotection strategy for assembling oligosaccharide libraries was reported previously (Scheme 1).[6] For example, regioselective silylation of the thiogalactopyranoside **1** with *tert*-butyldiphenylsilyl chloride (TBDPSCl) and imidazole furnished 2,3,4-triol **2** (100%), into which was regioselectively introduced a *p*-methoxybenzyl (PMB) group at the 3-O-position by benzylation mediated by tin ether to yield 2,4-diol **3** (49%) in two steps. 2-*O*-Chloroacetylation of compound **3** led to the desired acylated product **4** (52%) with good regioselectivity. Treatment of the 4-hydroxy compound **4** with levulinic acid, dicyclohexyl carbodiimide (DCC), and 4-(*N*,*N*-dimethylamino)pyridine (DMAP) gave the fully protected



Scheme 1.



Chi-Huey Wong is President of Academia Sinica and Professor of Chemistry at the Scripps Research Institute. His research interests are in the areas of bioorganic and synthetic chemistry, including the development of chemical syntheses based on enzymatic and chemoenzymatic reactions, the synthesis of complex carbohydrates, glycoproteins, and small-molecule probes for the study of carbohydrate-mediated biological recognition, the development of carbohydrate microarrays for high-throughput analysis of carbohydrate–protein interaction, and drug discovery.

Chung-Yi Wu received his PhD from the National Chiao-Tung University, Taiwan, in 2000. After postdoctoral research with Prof. Chi-Huey Wong at the Institute of Chemistry, Academia Sinica, and The Scripps Research Institute, he then joined the Genomics Research Center, Academia Sinica as an Assistant Research Fellow in 2006. His current research is concerned with the development of glycan arrays and carbohydrate-based vaccines.

Shang-Cheng Hung obtained his PhD from the National Tsing Hua University of Taiwan in 1992. After postdoctoral research with Prof. Andrew Streitwieser at the University of California at Berkeley and with Prof. Chi-Huey Wong at the Scripps Research Institute, in 1998 he started his independent research at the Institute of Chemistry, Academia Sinica. He moved to the Department of Chemistry, National Tsing Hua University in 2005. His research focuses on carbohydrate chemistry and chemical biology, including the development of regioselective one-pot protection and the synthesis of heparin oligosaccharides and mycobacterial cell envelope components.

Che-Hsiung Hsu received his BSc in Chemical Engineering from the National Cheng Kung University in 2002, and his MSc in Structural Biology from the National Tsing Hua University in 2005. Since 2006, he has been a PhD student at Academia Sinica in Taipei under the guidance of Prof. Chi-Huey Wong. His doctoral research focuses on the development of synthetic sialoside microarrays for the study of glycan-binding proteins.

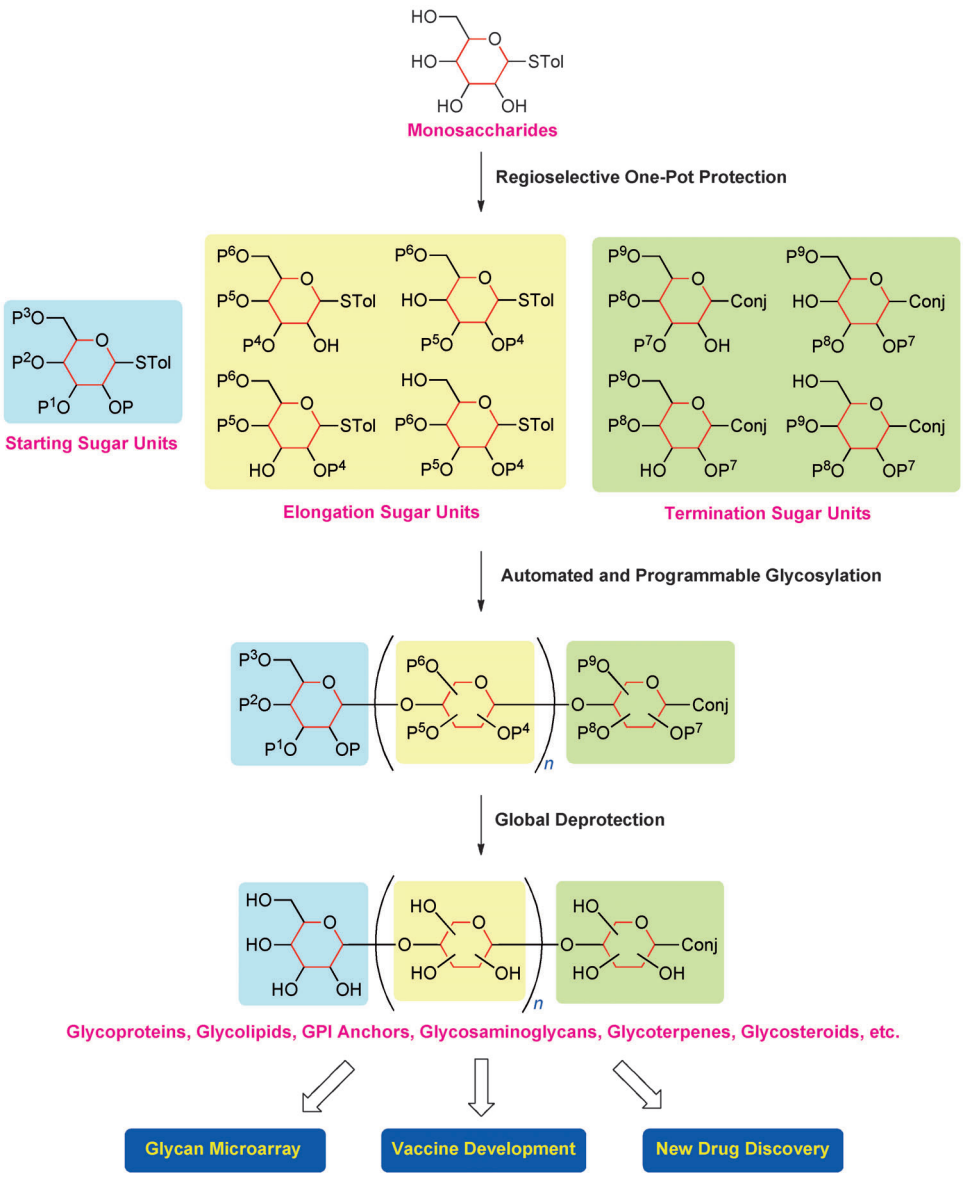


Figure 1. Straightforward routes for the synthesis of carbohydrates through a combination of "regioselective one-pot protection" and "automated and programmable glycosylation", and their application to the assembly of glycan microarrays, the development of vaccines, and the discovery of new drugs.

glycosyl donor **5** (83%), which was coupled with methyl 6-hydroxyhexanoate to afford the β-linked derivative **6** in 85% yield. The orthogonal protecting groups of compound **6** could be regioselectively cleaved through different reaction conditions to provide the free hydroxy groups for the synthesis of an oligosaccharide library.

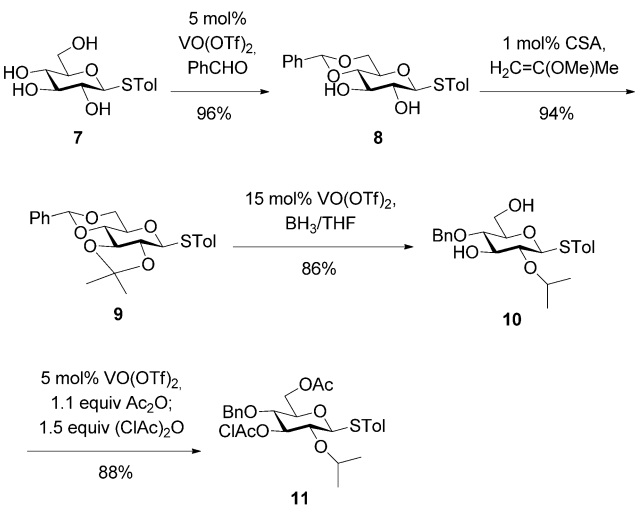
Chen et al.[7] described a direct formation of a 4,6-*O*-benzylidene acetal from tetraol **7** and benzaldehyde catalyzed by water-tolerant and recoverable $VO(OTf)_2$ at ambient temperature to give 2,3-diol **8** in excellent yield (96%; Scheme 2). The reaction conditions could be applied to a wide range of sugar substrates. Ketalization of alcohol **8** with 2-methoxypropene in the presence of 1 mol% camphorsulfonic acid (CSA) yielded 2,3-*O*-isopropylidene derivative **9** (94%), which underwent $VO(OTf)_2$-catalyzed regioselective reductive ring opening of the benzylidene acetal and isopropylidene ketal by borane to furnish 3,6-diol **10** (86%). The same catalyst was used for the regioselective acylation of compound **10** with different acid anhydrides, and the corresponding ester **11** was obtained in 88% yield in a one-pot manner. Thus, the thioglycoside **7** was regioselectively protected with the isopropyl, chloroacetyl, benzyl, and acetyl groups at the 2-O-, 3-O-, 4-O-, and 6-O-positions, respectively.

In addition, many new methods have been developed to selectively introduce one or more protecting groups onto a monosaccharide for further manipulation. These include, for example, organocatalytic 4-*O*-acylation of *n*-octyl β-D-glucopyranoside,[8] Me_2SnCl_2-catalyzed regioselective benzoylation of methyl α-D-glucopyranoside,[9] regioselective trisilylation of methyl α-glycosides catalyzed by an iridium complex,[10] tandem selective chlorination/*O*-formylation of sugars with Vilsmeier reagent,[11] and tandem acetalation/acetylation of sugars to introduce the isopropylidene and acetyl groups, simultaneously, under solvent-free conditions.[12]

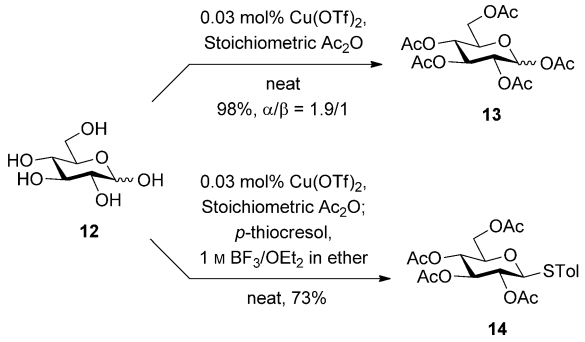
2.2. One-Pot Protection Strategy

2.2.1. One-Pot Per-O-acetylation

Per-*O*-acetylation of sugars is a fundamental reaction to prepare sugar synthons. Typically, large quantities of acetic anhydride and pyridine are used for the transformation. To promote green chemistry, an alternative procedure was reported in which only 0.03 mol% $Cu(OTf)_2$ catalyzed the per-*O*-acetylation of D-glucose (**12**) in the presence of a stoichiometric amount of acetic anhydride under solvent-free conditions to give the pyranosyl pentaacetate **13** (98%, $\alpha/\beta =$ 1.9:1) exclusively (Scheme 3).[13] This Lewis acid activated esterification is suitable for a variety of hexoses, and the corresponding derivatives are generated in excellent yields. In contrast to the conventional per-*O*-acetylation, which



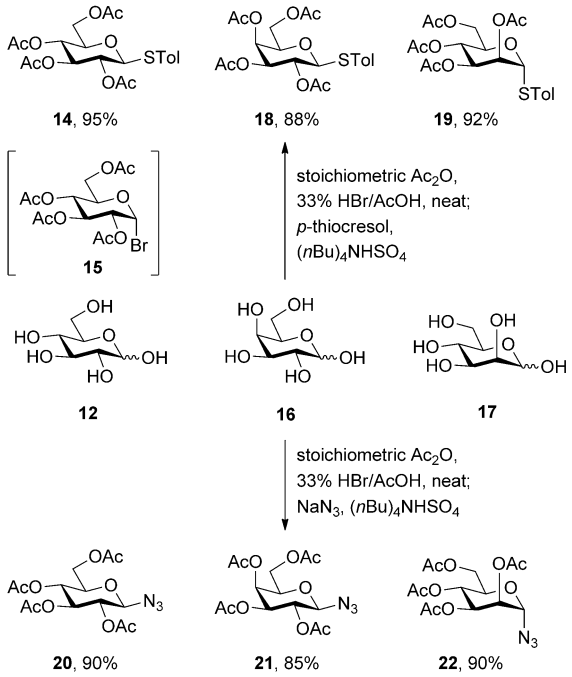
Scheme 2.



Scheme 3.

requires an excess amount of Ac_2O and subsequent neutralization followed by elaborate tedious workup and purification, this method, in which a stoichiometric amount of Ac_2O is employed, carries out the solvent-free sequential per-*O*-acetylation and anomeric substitution of hexoses in a one-pot manner. As in per-*O*-acetylation of D-glucose (**12**; or other hexoses), *p*-thiocresol and 1M $BF_3{\cdot}OEt_2$ in diethyl ether were added sequentially to the reaction solution at room temperature without additional solvent, and the desired thioglucoside **14** was isolated in 73% yield.

A generalized procedure for the one-pot synthesis of thioglycosides and glycosyl azides directly from unprotected reducing sugars under phase-transfer conditions begins with treating D-glucose (**12**) with a stoichiometric amount of Ac_2O and 33% HBr in AcOH (Scheme 4).[14a] Further addition of *p*-thiocresol and $(n\text{Bu})_4NHSO_4$ gave the thioglucoside **14** in 95% yield. The reaction proceeded through the α-glycosyl bromide intermediate **15**, which after nucleophilic substitution with *p*-thiocresol afforded the β-anomeric product. Applications of these conditions to D-galactose (**16**) and D-mannose (**17**) yielded the corresponding β-thiogalactoside **18** (88%) and α-thiomannoside **19** (92%), respectively. To extend the scope of nucleophiles, sodium azide was used instead of *p*-thiocresol, and the 2,3,4,6-tetra-*O*-acetylated

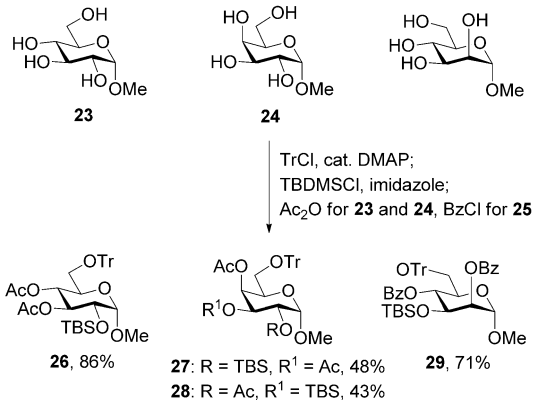


Scheme 4.

glycosyl azides **20**–**22** were obtained in 90%, 85%, and 90% yields, respectively. A similar transformation for the one-pot synthesis of thioglycosides from the corresponding hexoses through the glycosyl iodide intermediates has also been reported by Field and co-workers.[14b]

2.2.2. *One-Pot Tritylation/Silylation/Acylation*

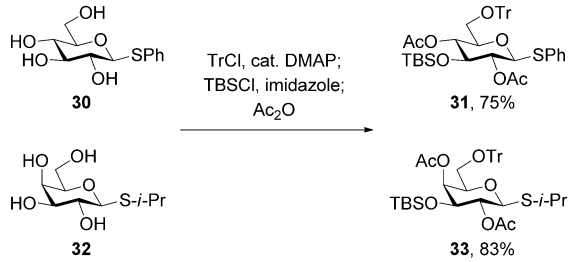
A one-pot tritylation/silylation/acylation of glycosides was carried out by DMAP-catalyzed 6-*O*-tritylation of methyl D-glucopyranoside **23** with trityl chloride followed by regioselective 2-*O*-silylation and subsequent 3,4-diacetylation to give the fully protected derivative **26** (86%) in a one-pot manner (Scheme 5).[15] However, treatment of methyl D-galactopyranoside **24** to a similar protection strategy furnished two products **27** (48%) and **28** (43%), because of the low



Scheme 5.

regioselectivity at the 2-*O*- and 3-*O*-positions during the silylation step. In the case of methyl D-mannopyranoside **25**, regioselective silylation preferred the less-hindered 3-*O*-position, and the 2,4-dibenzoate **29** was obtained in 71% yield.

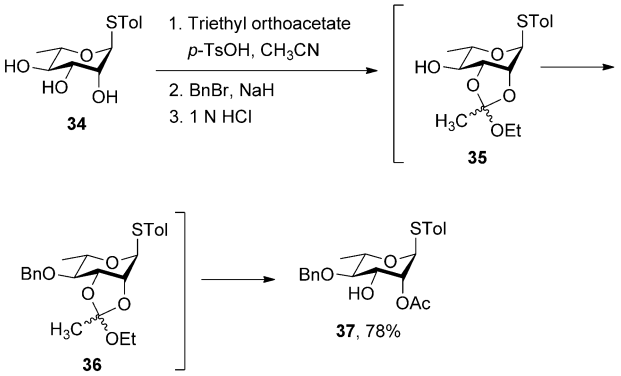
Compared to α-methyl glycosides **23** and **24**, the three-step one-pot transformation with the β-thioglycosides **30** and **32** (Scheme 6)[15] gave a very different result. In this case, both examples favored 3-*O*-silylation in a highly regioselective manner after installation of the trityl group at 6-*O*, and the 2,4-diacetates **31** and **33** were obtained in 75% and 83% yields, respectively.



Scheme 6.

2.2.3. One-Pot Orthoesterification/Benzylation/Orthoester Rearrangement

A simple one-pot method has been developed for the synthesis of partially protected mono- and disaccharide building blocks by using an orthoesterification/benzylation/orthoester rearrangement approach.[16] As described in Scheme 7, L-rhammopyranoside **34** was first treated with



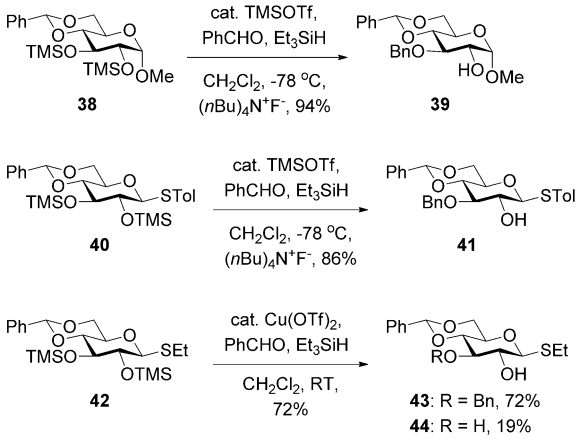
Scheme 7.

triethyl orthoacetate in the presence of *p*-TsOH as the catalyst to give the 4-hydroxy intermediate **35**, which was subjected to benzylation to furnish the fully protected intermediate **36**. Without quenching the reaction, 1N HCl was added to the same pot, and the orthoester underwent rearrangement to afford the 3-OH derivative **37** in 78% yield. This three-step protocol is particularly appropriate for sugars containing *cis*-dihydroxy moieties, such as D-mannopyranoside, D-galactopyranoside, L-fucopyranoside, and lactosyl derivatives.

2.2.4. Combinatorial and Regioselective One-Pot Protection

The preparation of fully protected hexopyranosides with a free hydroxy group at C2, C3, C4, or C6 from a hexopyranosyl 2,3,4,6-tetraol often suffers from: 1) an independent and multistep (4–6 steps) protection/deprotection sequence to synthesize each compound, 2) a tedious workup in each preparative step, 3) a time-consuming purification process to separate different regioisomers, and 4) a low yield of the expected product when the regioselectivity is poor. A novel idea to address these issues is to use a combinatorial, regioselective, and sequential one-pot procedure to differentially protect the 2-, 3-, 4-, and 6-hydroxy group of an anomeric α-OMe- or β-STol-bearing hexopyranoside. Such a method would be of vital importance to expedite the overall synthetic process and reduce the labor involved in the preparation of saccharides.

To test this idea, differentiation of two equatorial hydroxy groups at the 2-C- and 3-C-positions of glucosides were first investigated because the 4-C- and 6-C-hydroxy groups could be easily blocked by the benzylidene acetal (Scheme 8). A TMSOTf-catalyzed, highly regioselective, Et_3SiH-reductive

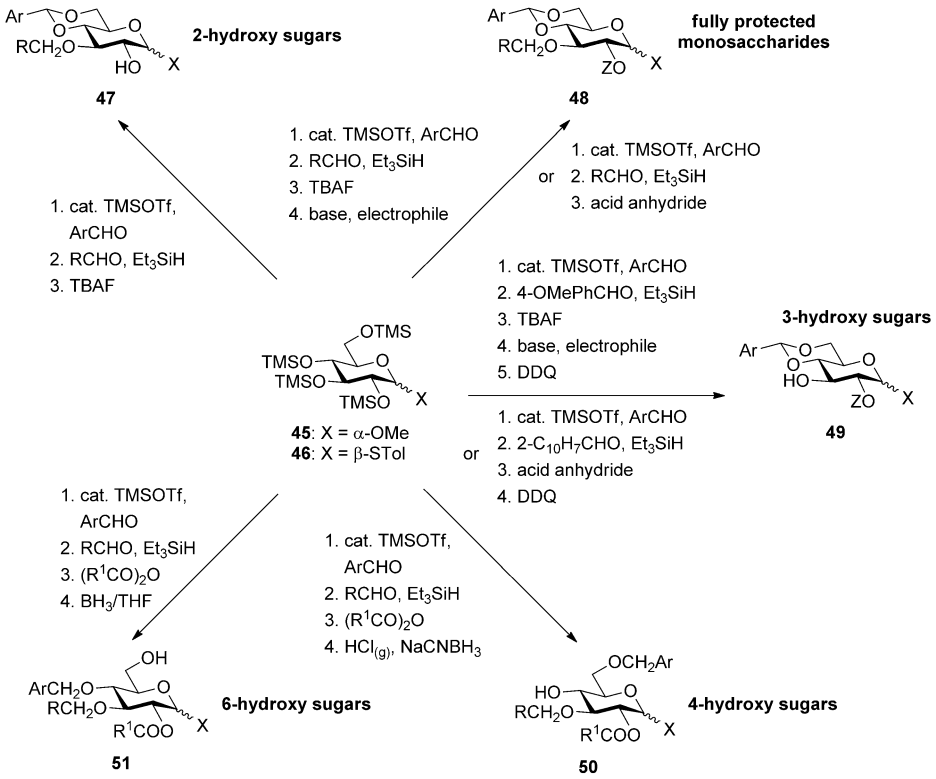


Scheme 8.

3-*O*-benzylation of the bis(TMS) ethers **38** and **40** with benzaldehyde at −78 °C afforded the 2-hydroxy derivatives **39** (94%) and **41** (86%), respectively.[17] The catalytic properties of different Lewis acids had been studied for the thioglucoside **42**, and the corresponding 2-hydroxy sugar **43** (72%) together with the TMS-hydrolyzed 2,3-diol **44** (19%) were isolated individually when $Cu(OTf)_2$ was used as the catalyst.[18] The combination of the above information led to TMSOTf being chosen for further investigation of the regioselective one-pot protection strategy.

The general protocols involved 1) selective protection of 4-O and 6-O as an arylidene acetal followed by regioselective reductive arylmethylation at 3-O to furnish the 2-hydroxy sugars; 2) 4,6-*O*-arylidenation, 3-*O*-arylmethylation, and sub-

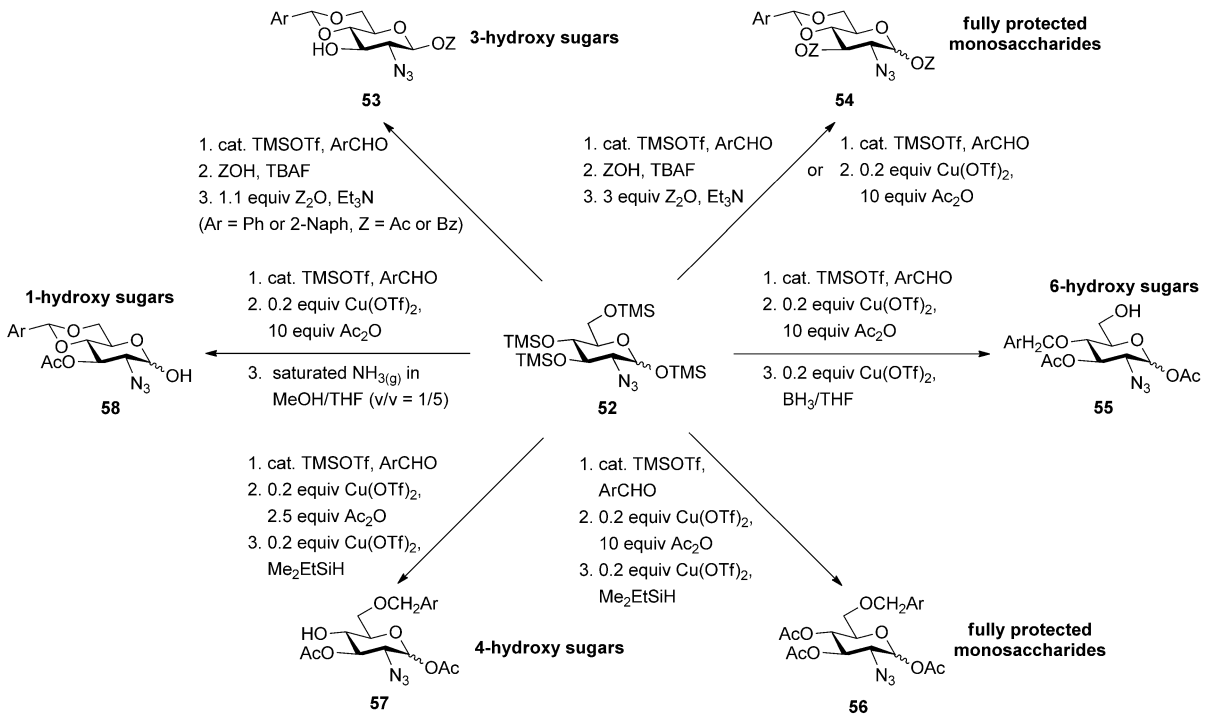
sequent etherification or acylation at 2-O to get the fully protected monosaccharides; 3) 4,6-*O*-arylidenation, temporary installation of PMB or 2-NAP at 3-O, etherification or acylation at 2-O, and final removal of the PMB or 2-NAP group to yield the 3-hydroxy sugars; and 4) 4,6-*O*-arylidenation, 3-*O*-arylmethylation, 2-*O*-acylation, and regioselective ring opening of the arylidene acetals at 4-O and 6-O to provide the 4-hydroxy and 6-hydroxy sugars, respectively.[19] As summarized in Scheme 9, starting from the 2,3,4,6-tetra-



Scheme 9.

O-trimethylsilylated glucosides **45** or **46**, TMSOTf first catalyzed the formation of an 4,6-*O*-arylidene acetal and a Et_3SiH-reductive 3-*O*-arylmethylation with the same or different aryl aldehyde (RCHO) to give a library of the 2-hydroxy sugars **47** after quenching by a stoichiometric amount of TBAF to remove the 2-*O*-TMS group. In another set of experiments, the first three steps were repeated, and the appropriate electrophiles were added under basic conditions to obtain the fully protected glycosides **48**, including the introduction of various benzyl, allyl, and acyl (Z) groups at the 2-O-position. Since TMSOTf could be used as a catalyst for the acylation reactions, the direct addition of various acid anhydrides to the 2-OTMS intermediate, which was generated after the first two steps, furnished the fully protected monosaccharides **48**. For the synthesis of the 3-hydroxy sugars **49**, PMB and 2-NAP were used as the temporary protecting groups at the 3-O-position. The same operation was repeated to prepare the fully protected glycosides **48**, and DDQ was finally added to the same flask to provide the desired products **49**. The fully protected glycosides **48**, generated by tandem 4,6-*O*-arylidenation, 3-*O*-arylmethylation, and acidic 2-*O*-acylation of **45** and **46**, could be used to synthesize the 4-hydroxy sugars **50** and 6-hydroxy sugars **51**. Regioselective ring opening of the arylidene acetals at 4-O and 6-O in the same pot by using $HCl_{(g)}/NaBH_3CN$ and BH_3/THF[20] as reductants yielded the expected compounds **50** and **51**, respectively. Thus, the simple per-*O*-trimethylsilylated pyranosides with anomeric α-OMe (**45**) and β-STol (**46**) could be efficiently transformed into hundreds of building blocks bearing different protecting groups.

D-Glucosamine and its *N*-acetylated and *N*-sulfonated derivatives are found as basic components in numerous biologically potent molecules, such as the cell-surface glycosylphosphatidylinositol (GPI) anchors, glycosphingolipids (Lewis a/x), *N*-glycoproteins, proteoglycans (heparan sulfate and heparin), hyaluronic acid, blood group antigens, bacterial cell wall, lipopolysaccharides, and chitin/chitosan. Continuing the above regioselective one-pot protection strategy, the anomeric nonfixed D-glucosamine-derived TMS-ether **52**, which was prepared from commercially available D-glucosamine in two steps, was used to synthesize a series of synthons, including fully protected monosaccharides and 1-, 3-, 4-, and 6-OH compounds. The azido group was selected to mask the 2-C-position of the glucosamine unit, because it could not only be used to control the α- or β-glycosidic linkage but could also be converted into an *N*-acetyl or free amino group. As illustrated in Scheme 10,[21] the 3-OH compounds **53** were produced after TMSOTf-catalyzed 4,6-*O*-benzylidenation of the TMS ether **52** followed by removal of the TMS groups with TBAF and regioselective 1-*O*-acylation[22] with Et_3N and 1.1 equiv of an acid anhydride. When the amount of acid anhydrides was increased up to 3 equiv, the 1,3-di-*O*-acylated products **54** were obtained in good yields. Without cleaving the TMS groups in the second step, the 4,6-*O*-benzylidene acetal intermediates were directly diacetylated by adding 20 mol% $Cu(OTf)_2$ and 10 equiv of Ac_2O to afford the fully protected monosaccharides **54**. In the same pot, the diacetate compounds could undergo BH_3- and Me_2EtSiH-reductive ring opening of the benzylidene acetal at 6-O and 4-O to yield the 6-hydroxy sugars **55** and 4-hydroxy sugars **57**, respectively. In the case of the 4-*O* ring cleavage, the amount of Ac_2O used in the second step influenced the products obtained. The 4-hydroxy compounds **57** were obtained when 2.5 equiv of Ac_2O were used, whereas



Scheme 10.

10 equiv yielded the further 4-*O*-acetylated product **56**. The same two-step operation of compound **52** was repeated, and the diacetate intermediates were treated with saturated $NH_{3(g)}$ in MeOH/THF to afford the expected 1-hydroxy compound **58** in a one-pot manner. Thus, the simple tetra-TMS ether **52** could be efficiently transformed into various synthons containing chemically differentiable protecting groups by this regioselective one-pot protection protocol.

3. Programmable One-Pot Oligosaccharide Synthesis

3.1. One-Pot Glycosylation

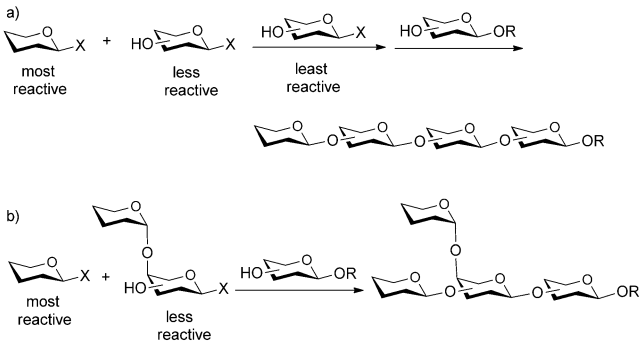
Recent advances in carbohydrate synthesis have greatly reduced the labor needed for preparing glycan materials. These include one-pot glycosylation, wherein oligosaccharides are made without isolation or purification of intermediates. Three general one-pot strategies are utilized for this purpose: 1) the chemoselective strategy,[23] in which the more reactive donor is selectively activated and treated with the less-reactive donor to provide a new glycoside which can subsequently react with the least reactive donor; 2) the preactivation strategy,[24] in which the donor is activated alone to generate an intermediate, which is subsequently coupled with the second donor bearing an identical aglycon at the reducing end; and 3) the orthogonal strategy,[25] in which the leaving group of one donor is selectively activated over another. There have been several excellent reviews recently that describe one-pot glycosylation methods.[26] In this part of the Review, we focus on the development of "programmable one-pot oligosaccharide synthesis" based on the chemoselective strategy, which has the potential to become an automatic process.

3.2. Strategies and Relative Reactivity Values (RRVs)

Carbohydrate chemists usually have to make huge efforts to manipulate protecting groups when they synthesize a target oligosaccharide containing several monosaccharide units. In contrast, the reactivity of the glycosyl donor and acceptor can be differentiated by protecting-group manipulation. This leads to chemoselective glycosylation, wherein only the more-reactive glycosyl donor is activated in the presence of a less-reactive one bearing an identical leaving group. The reaction product, therefore, can be consumed directly as the intermediate during the course of the one-pot reaction without further alteration of the aglycon. The Fraser-Reid research group first described the "armed/disarmed" concept, wherein the sugars bearing 2-*O*-ether ("armed") protecting groups were shown to react preferentially over those with 2-*O*-ester ("disarmed") groups.[27] The Ley research group later developed a new approach wherein a "semi-disarmed" state could be accomplished by using diacetal-protected thioglycoside glycosyl donors, thus providing an intermediate state of reactivity control.[28] They also provided the first demonstration that the reactivity of the glycosyl donor can be "tuned" quantitatively through protecting-group manipulation.

Before developing a programmable, high-speed automated synthesis with minimum protecting group manipulation, there must be a significant pool of saccharide units with well-defined reactivity and building blocks that afford simple,

branched carbohydrates with both α and β linkages.[29] The typical one-pot reactions start at the nonreducing end and proceed to the reducing end by taking advantage of the reaction profile, wherein the most reactive donor is added first and the least last (Scheme 11). For this strategy to



Scheme 11. The chemical strategy for the programmable one-pot synthesis of a) linear and b) branched oligosaccharides.

succeed, it is crucial to have information on the "relative reactivities" of a variety of glycosides. Previously, most one-pot syntheses were carried out by making use of the known difference in the reactivity between ether and ester protecting groups. In principle, a greater diversity of targets could be prepared if a library of reactivity values was available. Thus, a competitive HPLC experiment was developed to obtain and compare reactivity information from various glycosyl donors.[29] In this system, *p*-methylphenyl thioglycosides (STol) were chosen because they are easy to prepare, suitable for all monosaccharides, stable toward carbohydrate protecting group manipulations, and offer a convenient spectroscopic handle for measuring their reactivity by HPLC.[30] The relative reativity values (RRVs) were calculated by normalizing to the least reactive donor, peracetylated tolylthiomannoside (with RRV = 1.0). Hence, larger RRVs represent greater reactivity.[29] Currently, there are more than 400 building blocks in the database. Selected data are shown in Table 1.

Several interesting trends were revealed from the relative reactivity values of thioglycoside buiding blocks (Table 1):[29]

1. Pyranosides show reactivities that differ as a function of the sugar. The reactivity of commonly protected pyranosides (i.e., perbenzylated) decreases in the following order, fucose > galactose > mannose > glucose > sialic acid. Fucose is approximately fourfold more reactive than galactose, which is approximately sixfold more reactive than glucose. In addition, 2-hydroxymannose thioglycosides are, in general, much more reactive than the corresponding 2-protected derivatives.[29] These observations are consistent with the rates of hydrolysis of their corresponding glycosyl halides and glycosides.[31]

2. The reactivity of amino sugars can be tuned by the N-protecting groups. In the case of D-glucosamine and D-galactosamine, the reactivity decreases in the order NHCbz > NHTroc > NHPhth > N_3 > NHAc. Amino sugars bearing phthalimide groups show very low reactivity (RRV = 1.0–3.5) compared with Troc-bearing compounds (28.6). However, the range of reactivities (1–28.6) is still small given the large effect of the C2 group on the overall reactivity of a donor.

3. The protecting groups greatly influence the reactivity properties. The C2 substitution on galactose plays a significant role in deactivating the pyranose. The reactivity is most reduced by OClAc > OBz > OAc > OBn ⩾ OH > OSilyl > H. This phenomenon largely comes from the electronic effect. One elegant review by Fraser-Reid et al. described protecting-group effects in great detail.[32] The commonly accepted belief is that benzylated derivatives are always significantly more reactive than their benzoylated counterparts. In addition, the overall glycosyl donor reactivity is presumed to be directly correlated with the total number of benzyl substituents.[31a] However, recent studies reported that the donor reactivity of a 2-*O*-benzoylated *S*-benzoxazoyl glycoside is significantly higher than its 2-*O*-benzylated counterpart (Figure 2).[33]

4. The position that affects pyranoside reactivity the most is not always the same for all sugars. For example, the Ley research group reported that the C2-position had the greatest effect on the reactivity of mannose (followed by C6 > C4 > C3), while for galactose, the order is C4 > C3 > C2 > C6 from their RRVs.

5. *The magnitude of any protecting group reactivity effect is influenced by its position on the pyranoside.* While protecting groups affect the reactivity, the magnitude of this effect depends in most cases on the position of the group.[28] The effect can be demonstrated by comparing the reactivity of thiogalactoside with a benzoyl protecting group versus those with a hydroxy group at the same position. The reactivity increases as the hydroxy group is available in the series C6 (13.1) < C2 (17.6) < C3 (28.9) < C4 (67.1) compared to per-*O*-benzoylated thiogalactoside (5.7). The largest increase in the reaction rates caused by removal of the benzoyl group from the 4-O-position might be explained by the participation of the 4-O group in the destabilization of the putative cationic transition state.[31a] Since the effects of individual protecting groups on a sugar are not additive, it is difficult to predict the reactivity of a building block with several protecting groups.

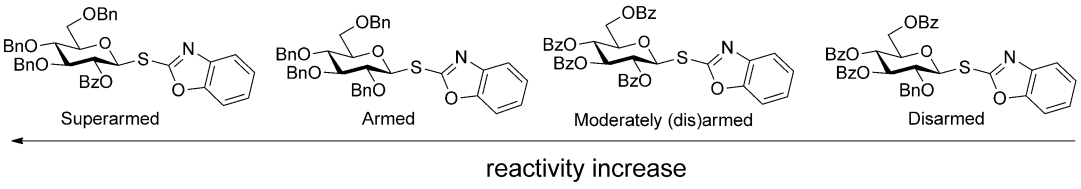
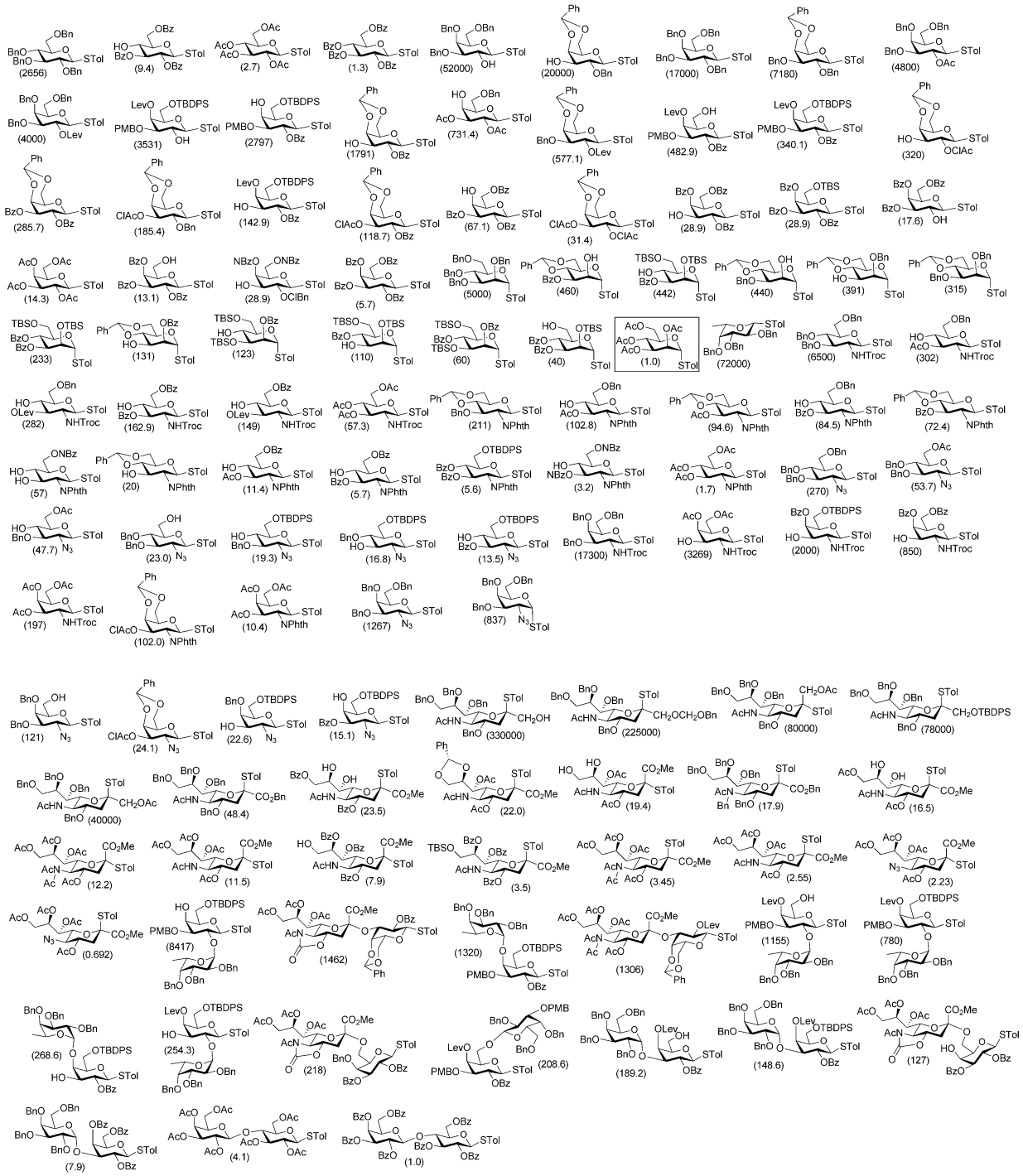


Figure 2. Increase in reactivity by introduction of a benzoyl group at C2.

Table 1: Relative reactivity values (RRVs) of thioglycosides for the programmable one-pot oligosaccharide synthesis.



6. Conformational effects (torsional effects). More *O* substituents at the axial position increases the reactivity of thioglycoside donors. Recently, the research groups of Bols[34] and Yamada[35] independently used bulky silyl protecting groups to force the oxygen substituent into an axial position and generate a twisted boat conformation to arm glycosyl donors (Figure 3). Bols and co-workers also calculated the reactivity of these "superarmed" donors and incorporated them into the reactivity-based one-pot synthesis of trisaccharide **62** in 64% yield (Scheme 12).

7. Influence of leaving groups. a) Steric effects: Altering the size of the anomeric group can tune the reactivity of the glycosyl donor. Boons et al.[36] discovered the influence of steric effects of thioglycosides on glycosyl reactivity and demonstrated that the anomeric reactivity of glycosyl donors or acceptors can be regulated by the bulkiness of the leaving

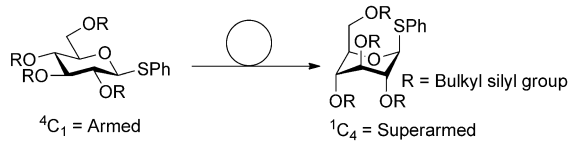
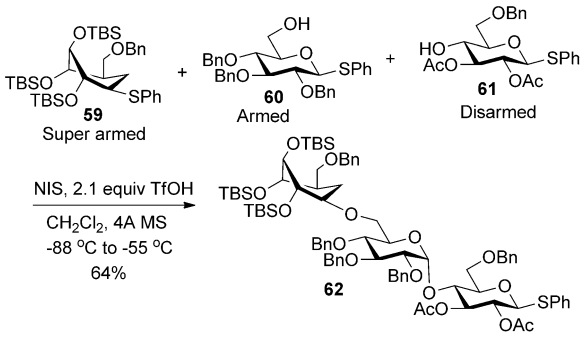


Figure 3. More O substituents in the axial position increase the reactivity of thioglycoside donors.

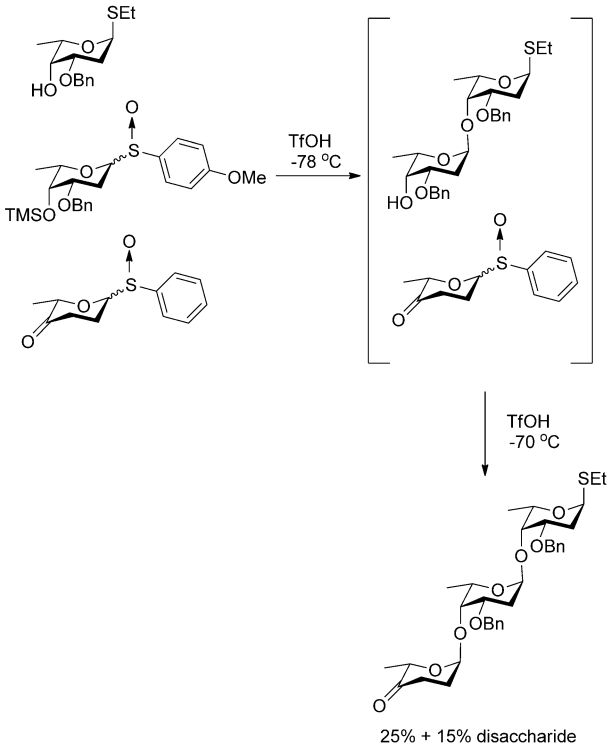


Scheme 12. Incorporation of the "superarmed" donor into a reactivity-based one-pot glycosylation.

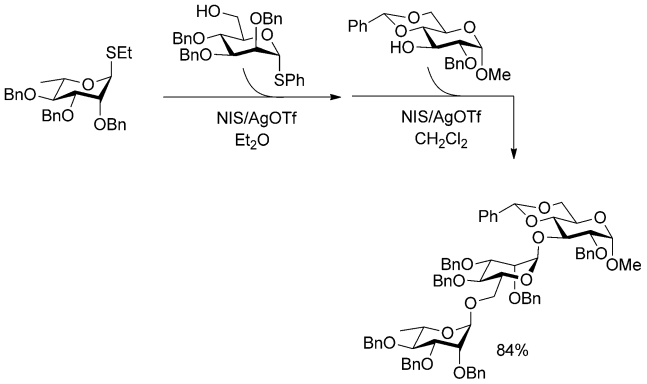
group. b) Electronic effects: The reactivity of the glycosyl donor can be influenced by *para* substitution of the phenyl ring, with the order of their reactivities being: OMe > H > NO_2. The difference in reactivity between OMe and H is sufficient for ciclamycine O trisaccharide to be synthesized in a one-pot glycosylation (Scheme 13).[23a]

More recently, Huang and co-workers systematically investigated the reactivity of the thioarylglycosides with various aglycon *para* substituents.[37] The reactivity trend for thioglycoside is: OMe > NHAc > N_3 > Br > NO_2.

8. Solvent effects: The reactivity of the donor and acceptor can be tuned by using different solvent systems. Lahmann and Oscarson introduced a one-pot two-step synthesis of trisaccharide, which can be achieved by performing the first glycosylation in Et_2O to activate the donor (low glycosylation rate) and the second in CH_2Cl_2/Et_2O (higher glycosylation rate; Scheme 14).[38]



Scheme 13. One-pot glycosylation of ciclamycine-*O* trisaccharide.



Scheme 14. Effect of solvent on the one-pot glycosylation.

3.3. OptiMer Software

In general, to achieve a convenient high-yielding coupling and minimize by-product formation, the difference in the quantitatively measured reactivity for each coupling should be larger than 100. A computer database, OptiMer, was created by Wong and co-workers to store the RRVs of many donor and donor/acceptor compounds (e.g., building blocks with one hydroxy group unprotected).[29] The database contains the name of the residue, the position of the unprotected hydroxy groups, and information on whether the C2 substituent directs the glycosylation in the α or β direction. The relative reactivity of each building block serves as an identity tag to identify or decode the structure of the target oligosaccharide. The database also stores the reference for the preparation of the compound as well as its structure. Once a user has selected an oligosaccharide structure, the program lists the best combination of building blocks for its preparation.

3.4. Reactivity-Based Programmable One-pot Oligosaccharide Synthesis

The programmable one-pot synthesis of oligosaccharides has the potential to affect many areas of drug discovery, as it provides access to complex carbohydrate structures. By using the OptiMer database, oligosaccharides containing three to six monosaccharides can be rapidly assembled in minutes or hours without intermediate workup or purification proce-

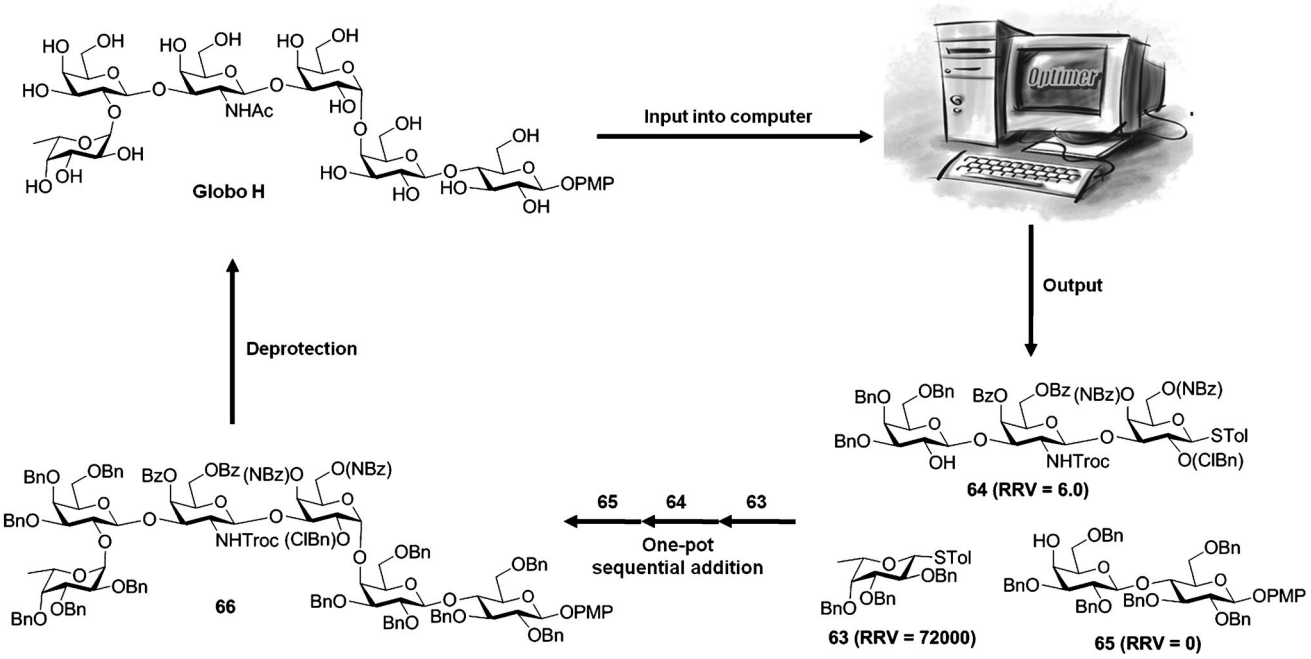
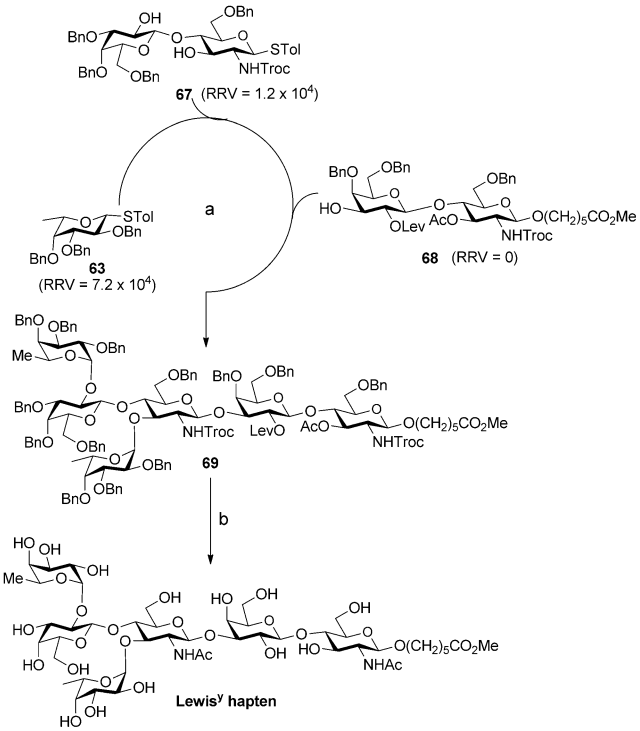


Figure 4. Programmable one-pot synthesis of oligosaccharides.

dures. For example, the hexasaccharide of globo H, a glycosyl ceramide found on various epithelial tumors, such as colon, ovarian, gastric, pancreatic, endometrial, lung, prostate, and breast cancers,[39] can be readily synthesized by a programmable one-pot method (Figure 4).[40] To generate a significant reactivity difference between the thioglycosides and control over the stereoselectivity of each glycosylation, the strategy for the synthesis of globo H saccharide involved the use of the three building blocks **63** (RRV = 7.2×10^4), **64** (RRV = 6), and **65** (RRV = 0). The reactivities were mainly tuned by electron-donating groups (benzyl ether and 2,2,2-trichloroethylcarbamate) and electron-withdrawing groups (benzoyl, *p*-nitrobenzoyl, and *o*-chlorobenzyl ethers). The intermediate trisaccharide **64** containing two β linkages was first created through a one-pot reaction.[40] Subsequently, through the sequential reaction of **63**, **64**, and **65** in the presence of NIS/TfOH as the promoter, the remaining two α linkages were built in another one-pot reaction to give the desired hexasaccharide **66** in 62% yield.

The especially high reactivity of the perbenzylated fucosyl donor **63** makes it an excellent reaction starting unit for the programmable one-pot syntheses of a range of fucose-containing oligosaccharides. For example, the carbohydrate-associated cancer antigen Lewis^y expressed on the surfaces of colorectal adenocarcinoma and heptacellular carcinomas can be prepared through the programmable one-pot approach (Scheme 15). Three building blocks with appropriate reactivity profiles were suggested by OptiMer: the fucosyl unit **63** (RRV = 7.2×10^4) and the two lactosaminyl units **67** (RRV = 1.2×10^4) and **68** (RRV = 0).[41] This one-pot synthetic operation was also performed using NIS/TfOH as a promoter (Scheme 15). The first glycosylation between the fucosyl donor **63** and the functional bridging lactosaminyl unit **67** was performed at −70 °C, whereas the second glycosylation



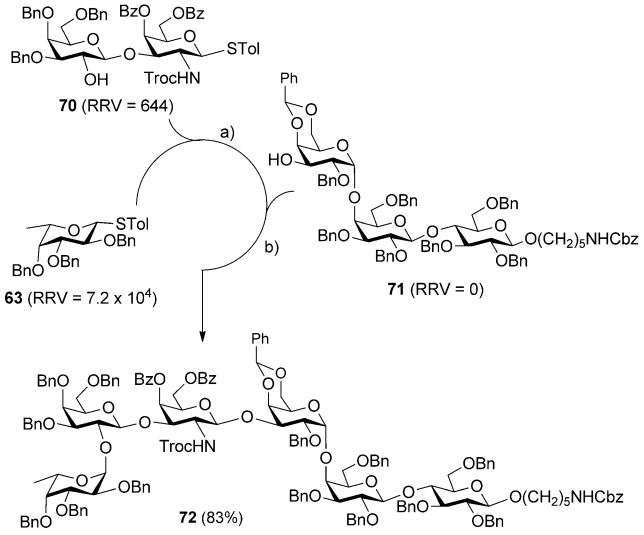
Scheme 15. Programmable one-pot synthesis of the Lewis^y hapten **69** and its deprotected compound. Conditions: a) 1. NIS, TfOH, M.S. AW-300, −70 °C; 2. **68**, NIS, TfOH, −25 °C, 44%; b) 1. Zn dust, Ac_2O; 2. NaOMe, MeOH/CH_2Cl_2; 3. Pd-black, H_2, MeOH/AcOH, 25% over three steps.

required a higher temperature (−25 °C). The lower temperature for the first glycosylation not only suppressed the formation of undesired by-product, succinimide, it also favored the formation of the α-glycosidic linkage. The

second glycosylation involved the coupling of two large sugar fragments and required a higher temperature for a practical reaction. The yield of fully protected determinant **69** was 44%, which was equivalent to 81% per glycosylation.

The programmable one-pot approach also represents an efficient combinatorial method suitable for the construction of a saccharide library.[26c] The method has been successfully applied to assemble designed linear and branched oligosaccharide structures, construct a 33-membered oligosaccharide library,[29,42] and diversify the sugar domain of natural products, such as aminoglycosides and vancomycin derivatives bearing different sugar substituents.[43]

The one-pot approach was also applicable to the study of the structure–activity relationships of complex oligosaccharides such as globo H. A refined [1+2+3] one-pot strategy in which the challenging Galα(1→4)Gal bond was formed in advance, at the stage of building block preparation, improved the yield of the one-pot synthesis of globo H from 62% to 83% (Scheme 16).[44] Based on the trisaccharide building



Scheme 16. Programmable one-pot synthesis of globo-H hexasaccharide by the [1+2+3] strategy. Conditions: a) NIS, TfOH, −40°C; b) NIS, TfOH, −30°C, 83% overall yield.

block **71**, which is valuable for the synthesis of all the globo family of oligosaccharides, a set of truncated globo H sequences were constructed and then arrayed covalently on glass slides for the fluorescence-based specificity analysis of the two monoclonal antibodies VK9 and Mbr1.[44] These glycans would also be useful in identifying optimal structure for the development of vaccines.

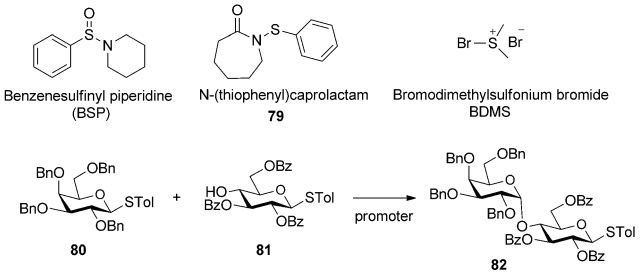
Additionally, the products of programmable one-pot synthesis can be used as substrates for the chemoenzymatic synthesis of extended structures such as the poly *N*-acetyl lactosamine oligomer, which is a precursor to a number of blood-group antigens.[45]

3.5. Promoters of the Programmable One-Pot Glycosylation

Programmable one-pot glycosylation normally utilizes *N*-iodosuccinimide/trifluoromethanesulfonic acid (NIS/TfOH) as the promoter system without problems. However, because one-pot glycosylation reactions usually involve multiple glycosylation steps, the succinimide generated by a stoichiometric amount of NIS unavoidably accumulates in the reaction mixture. Although the nitrogen atom of succinimide is a poor nucleophile, it can compete effectively with the highly unreactive acceptor for the remaining pool of activated glycosyl donors. This is particularly serious when the hydroxy group of the acceptor is highly hindered or has poor nucleophilicity. The efficiency of the activation of the thioglycoside donor by another thiophilic promoter dimethyl (thiomethyl) sulfonium trifluoromethanesulfonate (DMTST) is slower than that with NIS/TfOH.[29]

An alternative thiophilic reagent 1-(benzensulfinyl)piperidine/trifluoromethanesulfonic anhydride (BSP/Tf_2O) developed by Crich and Smith was thus utilized to solve these problems.[46] With a substoichiometric amount of BSP/Tf_2O as the promoter, the glycosylation of **81** (Table 2) with **63** resulted in a high yield, since the succinamide by-products

Table 2: Effect of different reaction promoters on the glycosylation of **80** and **81**.

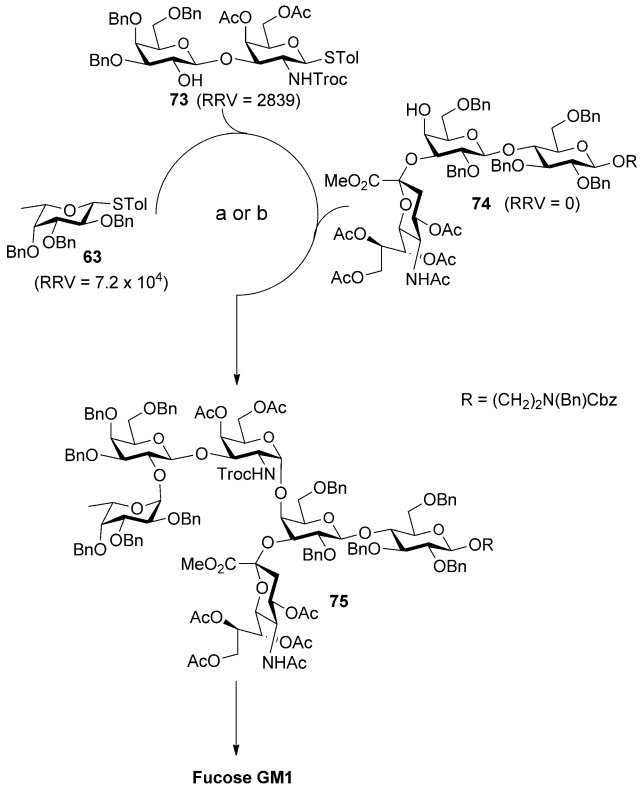


Entry	Promoter system	Yield [%]
1	*N*-(thiophenyl)caprolactam/Tf_2O	93[a]
2	*N*-(thiophenyl)caprolactam/Tf_2O	83[b,c]
3	benzenesulfinyl piperidine/Tf_2O	<15[b,d]
4	*N*-iodosuccinimide/TfOH	75[b]
5	bromodimethylsulfonium bromide/AgOTf	92[e]

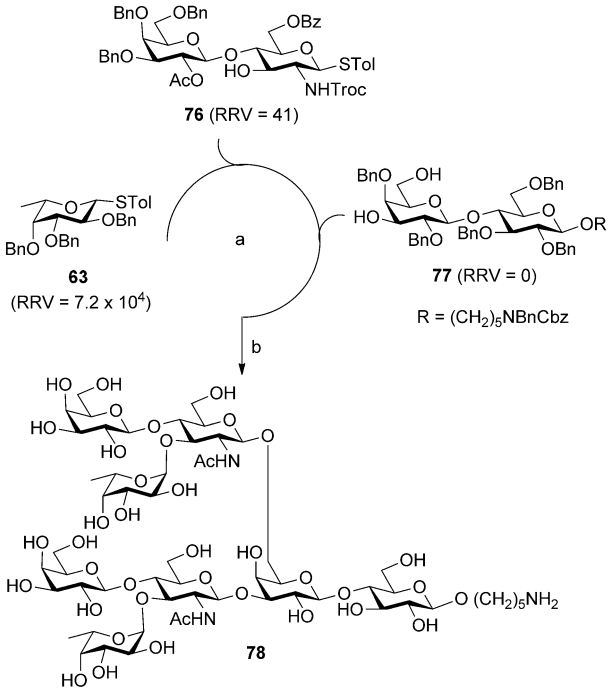
[a] Donor (1.1 equiv), acceptor (1.0 equiv), promoter (1.35 equiv), CH_2Cl_2, −78°C to −10°C. [b] 1:1:1 donor/acceptor/promoter, RT, 10 min. [c] A buffering reaction with TTBP gave comparable yields. [d] A rationalization is that BSP yields electrophilic by-products, which serve to further activate the products. When 0.5 equiv of BSP were used, the yield was 63%. [e] Donor (1.2 equiv), acceptor (1.0 equiv), BDMS (1.4 equiv), AgOTf (3.2 equiv), CH_2Cl_2/Et_2O 1:1, −35°C to RT.

produced by NIS/TfOH were not generated.[47] One possible rationalization may be that the reaction pathway produces sulfonium by-products, which can further promote glycosylation. The utility of BSP/Tf_2O for the programmable one-pot glycosylation has been demonstrated by the one-pot synthesis of fucosyl-GM1 (Scheme 17). When BSP/Tf_2O was used as the promoter, the overall yield was improved (from 13% to 22%), and the reaction time required was also reduced (from 1 day to 5 h) compared to the NIS/TfOH- and DMTST-promoted systems.[47]

Moreover, the programmable one-pot syntheses of the biologically important α-Gal pentasaccharide derivative[48]



Scheme 17. Programmable one-pot synthesis of fucosyl-GM1. Conditions for the NIS/Tf_2O- and DMTST-promoted one-pot reactions: route a) 1. NIS, TfOH, CH_2Cl_2, −70 °C, 36%; 2. **74**, DMTST, 0 °C, 36%. For the BSP/Tf_2O-promoted one-pot reaction: route b) 1. BSP, Tf_2O, CH_2Cl_2, −70 °C to −10 °C, 47%; 2. **74**, BSP, Tf_2O, CH_2Cl_2, −70 to 0 °C, 47%.

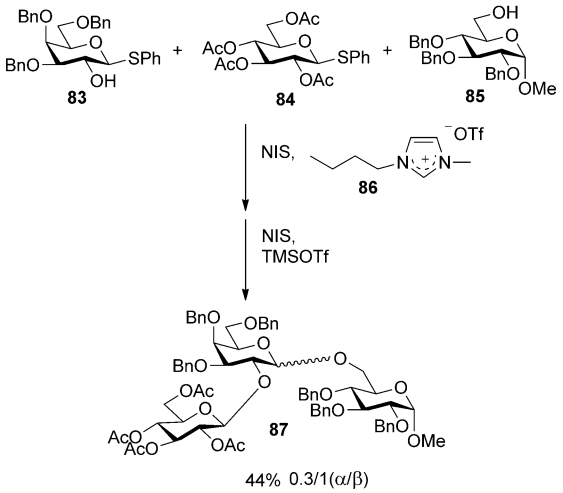


Scheme 18. Programmable one-pot synthesis of N3 minor antigen **78**. a) 1. BSP, Tf_2O, M.S. AW-300, CH_2Cl_2, −70 to −10 °C; 2. NIS, TfOH, 0 °C to RT; b) 1. Zn, AcOH, RT; 2. Ac_2O, DMAP, pyridine, 0 °C to RT; 3. NaOMe, MeOH, RT; 4. Pd-black, H_2 (1 atm), MeOH with 5% formic acid, 11% from step (a).

and tumor-associated antigen N3 minor octasaccharide **78** (Scheme 18)[49] were demonstrated by using the BSP/Tf_2O promoter system.

To improve the programmable one-pot glycosylation technology for oligosaccharide synthesis further, there is still a need for a better promoter, which must be thiophilic, amenable to the reactivity-based one-pot strategy, suitable over a broader temperature range, and most importantly must not generate by-products that will interfere with the reaction. It is known that benzenesulfenyl triflate is an extremely powerful thiophilic reagent.[50] Although it is a potent electrophile, its use is problematic due to its instability and the requirement for its in situ preparation from benzenesulfinyl chloride and silver trifluoromethanesulfonate. A new reagent is thus needed that complements benzenesulfenyl triflate and which is stable and conveniently accessible. Recently, *N*-(phenylthio)caprolactam (**79**) was identified as a new promoter for the selective activation of thioglycoside donors for glycosylation (Table 2).[51] Phenylthioamide **79** could react with Tf_2O to generate in situ an activated promoter, which subsequently activates the thioglycoside for glycosidic bond formation.[51] This promoter works exceptionally well; several disaccharides were accessed, including thioglycoside **82**, with yields up to 93% (Table 2, entry 1). Notably, glycosylation reactions at room temperature using this promoter were clean and fast (Table 2, entry 2). This was an improvement over other promoters for glycosylation at room temperature (Table 2, entries 3 and 4), although BSP/Tf_2O is still an excellent promoter for low-temperature glycosylation. The promoter **79** is adaptable to the reactivity-based one-pot synthesis of oligosaccharides, as demonstrated by the synthesis of Gb3 and *iso*Gb3 in 47% and 50% yield, respectively.[52]

Ye and co-workers recently applied bromodimethylsulfonium bromide (BDMS) as a novel thiophilic promoter for glycosylation. BDMS is effective for the activation of both "armed" and "disarmed" thioglycosides.[53] When BDMS/AgOTf was used, disaccharide **82** could be obtained in high yield with almost no by-products (Table 2, entry 5). This promoter is applicable to the reactivity-based one-pot synthesis of oligosaccharides, as demonstrated by the synthesis of the trisaccharide Galα(1→4)Glcβ(1→4)Glc.[53] The Galan research group recently reported the use of the ionic liquid 1-butyl-3-methylimidazolium triflate ([bmim][OTf], **86**) as a cosolvent and mild glycosylation promoter for thiophenyl and trichloroimidate glycosides at room temperature.[54] The potential of this ionic liquid promoter for reactivity-based one-pot reactions was demonstrated by a one-pot synthesis of a branched trisaccharide (Scheme 19).[55] With the mixture of donors **83** and **84** and acceptor **85**, the "armed" donor **83** was selectively activated and reacted with acceptor **85** by using **86** and NIS as the promoter and cosolvent to give a disaccharide intermediate. The "disarmed" thioglycoside donor **84** required the presence of a catalytic Lewis acid.[55] After the



Scheme 19. One-pot glycosylation of branched trisaccharides by using [bmim][OTf] as the promoter.

addition of NIS and a catalytic amount of TMSOTf, **84** was glycosylated with the disaccharide to give **87** in 44% yield.

3.6. Synthesis of Heparin-like Oligosaccharides

Heparin and heparan sulfate are structurally related linear polyanionic polysaccharides that belong to the glycosaminoglycan family. Both compounds are diversely sulfated and marked with extreme heterogeneity within the primary structure that is formed by an alternating 1→4-linked uronic acid (either D-glucuronic acid or L-iduronic acid) and D-glucosamine backbone.[56] A multitude of proteins recognize specific patterns along the sugar sequence, thus permitting local availability or altering the protein's state of activity.[57] Through these interactions, heparin and heparan sulfate play crucial roles in a number of biological events, many of which are biomedically important.[58] Mainly occurring in mast cells, heparin is a widely used clinical anticoagulant as a result of its high-affinity binding with antithrombin III, an important inhibitor of the blood-coagulation cascade.[59] On the other hand, heparan sulfate is ubiquitously distributed on the cell surface as a component of proteoglycans and mediates many physiological and pathophysiological processes, such as growth-factor regulation, viral and bacterial infection, inflammatory response, angiogenesis, tumor metastasis, cell adhesion, and lipid metabolism.[60]

A host of synthetic designs for the acquisition of chemically defined heparin-like oligosaccharides for use in studies on structure–activity relationships have been disclosed by many research groups. The majority of these preparations were purely chemical, but the latest advances have also utilized chemoenzymatic synthesis as an attractive alternative. Chemical approaches targeting the antithrombin-binding heparin pentasaccharide[61] and oligosaccharides composed of the major repeating unit of heparin[62] dominated early studies. The need for building heparin-like oligosaccharide libraries later prompted the progress in modular assembly strategies.[63] Continuous advances in carbohydrate chemistry increased the length of the oligosaccharides that could be obtained; however, the progress in this difficult area remained slow. A recent report described the synthesis of a dodecasaccharide[64]—the longest polymer achieved to date—with homogeneous repeating units (i.e., the major trisulfonated disaccharide found in heparin). The synthesis of a hexasaccharide appears to be the current limit, if variations in the repeating disaccharide are desired. Conversely, chemoenzymatic syntheses have the potential to generate longer structures.[65] With a prime source of the polymeric heparosan from *Escherichia coli* capsular polysaccharide K5,[66] these efforts relied on the enhanced specific activity of functional recombinant enzymes[67] and the aryl sulfotransferase coupled regeneration of 3′-phosphoadenosine-5′-phosphosulfate (PAPS)[68] that significantly reduced the cost of enzyme-mediated sulfonations. Functional groups could be introduced at target locations within the polymer skeleton by applying a strategic series of enzymatic transformations. These transformations had been conducted in solution,[69] on sensor chips,[70] and in a digital microfluidic chip analogous to a Golgi organelle.[71] Recently, Liu et al.[72] also reported a chemoenzymatic method capable of controlled chain elongation, selective *N*-sulfonation, and selective C5 epimerization.[72] The enzyme-based approach, however, suffers from the problem of product isolation, purification, and intermediate analysis.

The chemical syntheses of heparinlike oligosaccharides include several technical hurdles that set it apart from other sugar targets. L-Idose and its corresponding uronic acid are not available from natural sources; thus, methods such as stereochemical C5 inversion of the readily available D-glucose-based compounds[62c,73] and de novo synthesis[74] from 1,2-isopropylidene-α-D-xylofuranose have been developed. The numerous *O*-sulfonation and *N*-substitution sites, as well as the regio- and stereochemical requirement of the glycosidic bonds need to be considered in the selection of orthogonal protecting groups. The uronic acid derivatives were often used during the early stage of the synthesis to avoid the complicating oxidation of the D-glucose or L-idose component after the elongation process. Nonetheless, because the C5 carboxy group is prone to epimerization and uronic acid donors tend to have low reactivity, recent synthetic efforts[63b,c,75] involved the oxidation of the 6-hydroxy group of the D-glucoside or L-idoside components after the requisite glycosidic bonds were formed. The 1,2-*trans* glycosidic bond at the anomeric center of the uronic acid precursor is easily afforded by neighboring-group assistance of a C2 ester. However, the 1,2-*cis* orientation in the case of D-glucosamine (the α isomer) was achieved by the less-effective anomeric effect, with the amino group masked by an azide which may be transformed subsequently into another functional group. Fortunately, it was discovered that an axially oriented free hydroxy group in the glycosyl acceptor promotes an α-stereoselective formation of a glycosidic bond.[76] This phenomenon was exploited by Seeberger and co-workers through the use of acceptors locked at the $^1C_4$ conformation.[77]

With respect to the repeating disaccharide backbone of heparin, chain elongation is mostly performed by utilizing disaccharide building blocks through the convergent $[n+2]$ block approach. Strategies for the coupling of longer building blocks[62b,63a] have also been reported. A single repeating disaccharide building block such as those depicted in Figure 5 (**88**, **89**, and **90**) could be used to synthesize oligosaccharides

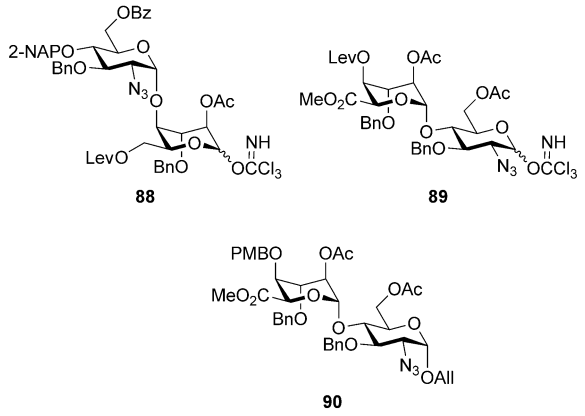
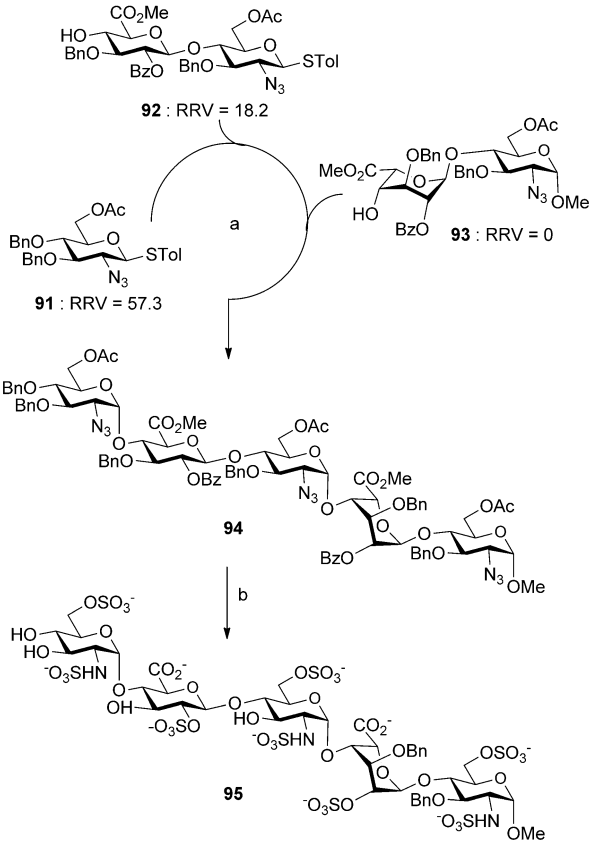


Figure 5. Examples of disaccharide building blocks utilized in the preparation of oligosaccharides comprised of the major repeating unit of heparin.

of various lengths comprised of the major repeating unit of heparin. Chain elongations by using compounds **88**[62c] and **89**[62d] could only be carried out from the reducing to the nonreducing end. Compound **90**,[78] on the other hand, could be readily transformed into either a donor or an acceptor for glycosylations in either direction with different donor and acceptor lengths.

A chain with a heparin backbone has been synthesized by programmable one-pot glycosylation, by utilizing thioglycoside building blocks with well-defined reactivity.[75a] Therein, the hydroxy groups set to carry the sulfonate moieties were protected as acetyl and benzoyl groups, whereas those that would be free were masked as benzyl ethers. The primary hydroxy groups that needed to be selectively oxidized were protected with a TBDPS group. After the initial glycosylations to form the disaccharide derivatives, the uronic acids were generated and protected as methyl esters to yield the disaccharides **92** and **93**. For the one-pot pentasaccharide synthesis (Scheme 20), azidoglucosyl donor **91** (RRV = 53.7) was first coupled with disaccharide acceptor **92** (RRV = 18.2). Then, the α-methyl disaccharide acceptor **93** was added to the reaction mixture to afford the fully protected pentasaccharide **94** in 20% yield. Finally, the heparin-like pentasaccharide **95** was produced after O- and N-sulfonations and global deprotection.

Oligosaccharide libraries are useful for identifying sugar structures that interact with proteins and cause a particular biological event. Although not all are known to exist, the structural variations in heparin and heparan sulfate account for 48 possibilities of component disaccharides.[79] Hung and co-workers synthesized the full set of 48 disaccharide building blocks (Figure 6), which could be readily used for the



Scheme 20. Programmable one-pot synthesis of heparin pentasaccharide. a) 1. NIS, TfOH, CH_2Cl_2, −45 °C to RT; 2. NIS, TfOH, CH_2Cl_2, −45 °C to RT, 20%; b) 1. LiOH, THF; 2. $SO_3{\cdot}Et_3N$, DMF; 3. H_2, Pd/C; 4. $SO_3{\cdot}Pyr$, H_2O, 33%.

generation of oligosaccharide libraries.[80] Chain elongations can be achieved by deprotection of the 2-naphthylmethyl (2-NAP) group to form the glycosyl acceptor and transformation of the 1,6-anhydro ring of the idose component into a glycosyl donor. Standard protecting-group patterns were used, and the acetyl and benzyloxycarbonyl protecting groups for the amine moiety were easily accessed from the azido group. Moreover, other research groups, such as those of Seeberger,[63a] Boons,[63b] and Huang,[63c] constructed other modular assemblies for a more specialized collection of oligosaccharides by using smaller sets of disaccharide building blocks (Figure 7). Several tetrasaccharides were assembled by Seeberger and co-workers from their disaccharides, but elongation to hexasaccharides required the use of trisaccharide modules. Boons and co-workers have successfully generated 11 tetrasaccharide constructs and 1 hexasaccharide from their building blocks by using the modular approach. These oligosaccharides were utilized in an inhibition study of BACE-1, a protease involved in the propagation of Alzheimer's disease. Moreover, Bonnaffé and co-workers suggested[81] a different approach, in which a single disaccharide donor was activated in the presence of different acceptors. The resulting mixture of oligosaccharides was then subjected to functional-group transformations, and the oligosaccharides were then separated on a reversed-phase column for further transformation.

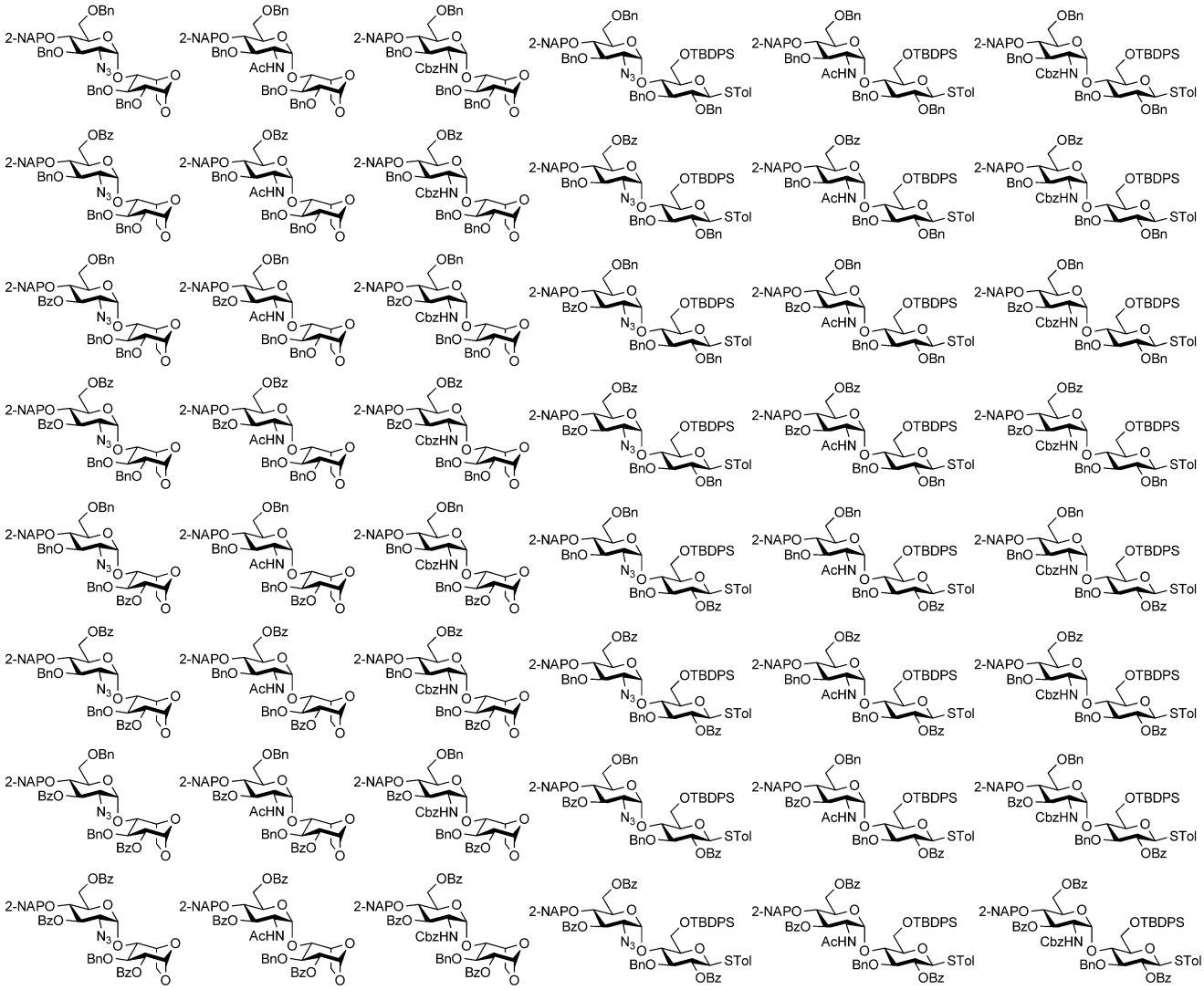


Figure 6. The 48 disaccharide building blocks prepared by Hung et al. for the assembly of heparin and heparan sulfate oligosaccharide libraries.

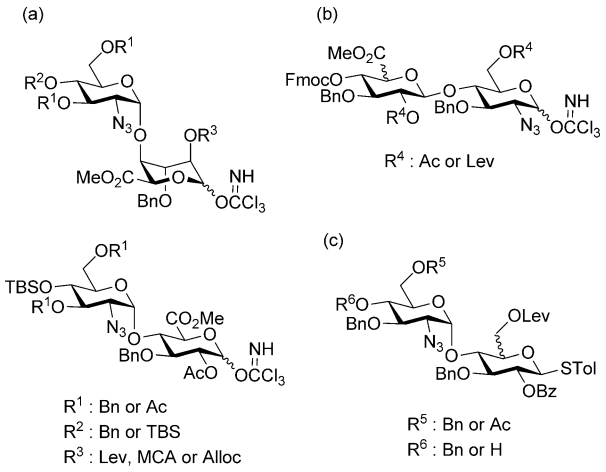
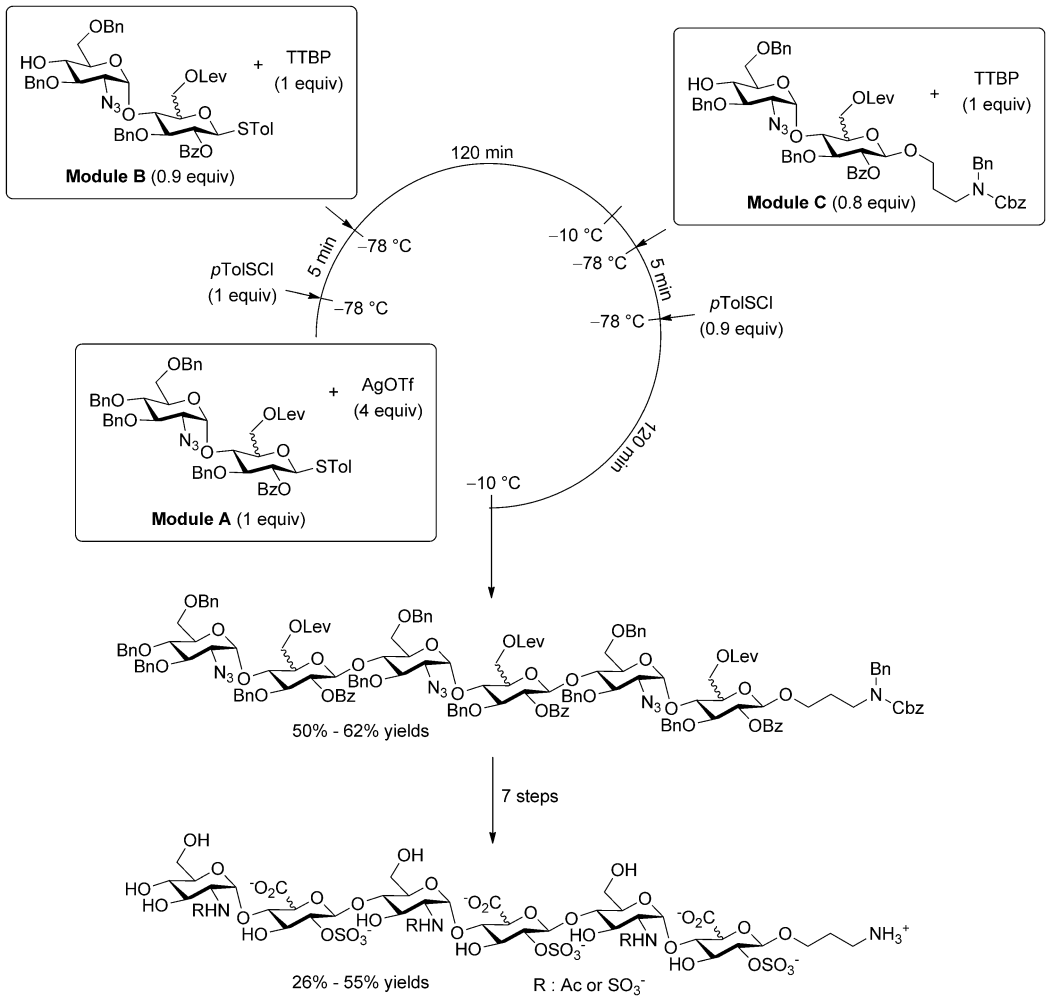


Figure 7. Disaccharide modules for the synthesis of heparin-like oligosaccharides with various disaccharide repeating patterns, as reported by the research groups of a) Seeberger, b) Boons, and c) Huang.

In contrast to previous efforts, Huang and co-workers incorporated the preactivation technique in their modular approach (Scheme 21), which permitted the one-pot assembly of several hexasaccharide skeletons without the use of the relative reactivity of the building blocks. The nonreducing end module (Module **A**) was premixed with and concomitantly activated by *p*TolSCl/AgOTf promoter. Module **B** was added next. By using 2,4,6-tri-*tert*-butylpyrimidine (TTBP) as an acid scavenger, the tetrasaccharide was presumably produced, which underwent activation and mixing with the reducing end block Module **C** and TTBP. The one-pot procedure, which was achieved in less than 5 h, delivered the required fully protected hexasaccharide in 50–62% yield. The target oligosaccharides were obtained in an overall yield of 26–55% after seven functional group transformation steps. Alhough many of the modular or one-pot syntheses lack the quantitative measurement of the building blocks for use in the automated design, the principles described could be useful for achieving this goal.



Scheme 21. Modular one-pot synthesis of heparin-like hexasaccharides by the preactivation-based combinatorial approach.

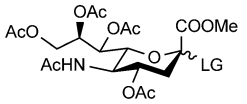
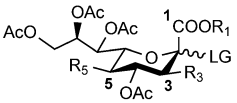
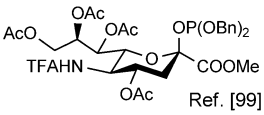
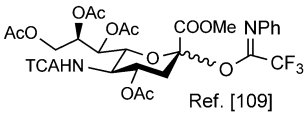
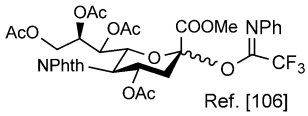
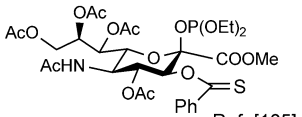
3.7. 4,5-Oxazolidinone-Protected Sialyl Donors for Efficient Syntheses of α-Sialosides

Sialic acids are a family of naturally occurring derivatives of neuraminic acid (5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid) commonly found in higher animals and certain microorganisms. Among more than 50 derivatives reported so far, C5 amino derivatives represent the major structural variations at which the amino function can be acetylated (Neu5Ac), glycolylated (Neu5Gc), or substituted with a hydroxy group (KDN).[82] There are also additional substitutions (e.g. *O*-acetyl) at the C4, C7, C8, and C9 hydroxy groups. Sialic acids are usually found joined to galactosides through the α(2→3) or α(2→6) linkage, *N*-acetylgalactosamine through the α(2→6) linkage, and other sialic acids through an α(2→8) or α(2→9) linkage at the outmost positions of the glycoproteins and glycolipids. The terminally exposed position on the cell surface allows conjugates containing sialic acid to be exploited as receptors for viruses and bacteria, in addition to governing a wide variety of important biological, pathological, and immunological processes.[83] The chemical synthesis of sialic acid glycosides can be achieved by the reaction of an oxocarbenium ion, generated from a properly protected sialic acid donor, with the sugar acceptor partner bearing one or more free hydroxy groups. The use of sialic acid donors, however, is complicated by its special structural features. The presence of an electron-withdrawing carboxy group at C1 reduces the reactivity of the anomeric center toward glycosylation. Alhough the sialyl donor with a protected hydroxymethyl group at the anomeric center is 1000 times more reactive than the normal ester-containing reagent (Table 1),[84] it provided only β-sialosides in the glycosylation. Meanwhile, stereochemical control of the α selectivity is a great challenge because of the absence of C3 functionality to direct the stereochemical outcome, which makes the reaction prone to elimination.

Over the past several years, significant efforts have been directed toward the development of sialic acid donors for efficient α-sialylation; the progress is summarized in several excellent reviews.[85] In short, the sialic acid donors prepared chemically possess the following three main characteristics: leaving-group optimization, structure modification, and optimized combination (Table 3).

The leaving-group optimization is mostly used for the anomeric leaving group, including halides, phosphites, sulfides, and *N*-phenyltrifluoroacetimidate. For these donors, the participation of acetonitrile is generally essential for augmenting the α selectivity, but the yield of the α product is normally no higher than 70%. Thioglycosides such as SMe and SPh in combination with the nitrile solvent are the most widely used. The next strategy is to introduce an auxiliary group at C1 or C3 as well as structure modifications at C5. Auxiliaries at C1 are generally less effective in directing α-selective sialylation reactions. The C3-modified sialyl donors normally display high α selectivity, although additional multi-

Table 3: Common sialic acid donors for chemical sialylation.

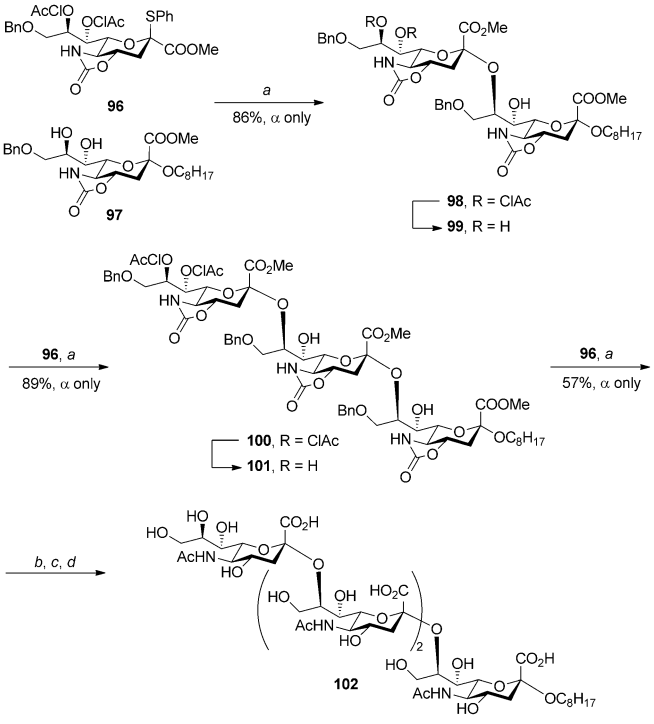
A) Leaving group (LG) optimization	B) Structure modification	C) Optimized combination
		 Ref. [99]
LG= 1. halides: Cl,[86] Br[86a]	C1 modification: R^1=C_2H_4SMe,[95] CH_2CONMe_2,[96] CH_2CN[97]	 Ref. [109]
2. phosphites, phosphates: $O(P)(OEt)_2$,[87] $O(P)(OBn)_2$,[88] $O(P)O(OBu)_2$[89]	C3 modification: R^3=OH,[98] Br,[99] O(C)SPh,[99] SPh,[100] SePh[101]	 Ref. [106]
3. sulfides: SMe,[90] S(C)SOEt,[50b,91] SPh,[92] SBox[93]	C5 modification: R^5=NAc_2,[102] NHTFA,[103] NHTroc,[104] N_3,[84a] NPhth,[105] NHTCA[104b,106] 4,5-carbamate,[89,107] 4,5-carbonate[108]	 Ref. [105]
4. *N*-phenyltrifluoroacetimidate: $OC(NPh)CF_3$[94]		

ple steps are required for the installation and removal of such an auxiliary group. The third strategy is to seek the optimized combination of the leaving group (e.g. phosophite or *N*-phenyltrifluoroacetimidate) and positional modification (e.g. C3 or C5) to prepare new sialyl donors for selective α-sialylation.

In the past decade, modification of the *N*-acetyl group at the C5-position of Neu5Ac donor has been shown to be exceedingly effective for α-selective sialylation (Table 3).[110] A recent development that shows potential is the use of cyclic protecting groups, including carbamate and carbonate, which have been previously shown to exhibit stereodirecting effects towards glycosylations for glycosides such as glucose and *N*-acetylglucosamine.[111] In 2006, Takahashi and co-workers first reported 5-*N*,4-*O*-oxazolidinone-protected thioglycoside of Neu5Ac **96**[107a] for the highly efficient synthesis of α(2→8)-linked oligosialic acids (Scheme 22). In this approach, the first reaction step was the coupling of 5-*N*,4-*O*-carbonyl-protected donor **96** and acceptor **97**, followed by the selective deprotection of the chloroacetyl groups at C7 and C8 of the resulting disialic acid **98**. The diol **99** that was formed was subsequently subjected to a coupling reaction with donor **96** to form trimer **100**. The desired α(2→8)-linked tetrasialic acid derivative **102** was obtained after further coupling and deprotection processes. Complete α selectivity was observed in every coupling reaction. The excellent α selectivity was achieved in dichloromethane without the assistance of a nitrile solvent. This approach was further applied to the convergent synthesis of the GP1c glycolipid epitope, which is one of the most complex c-series gangliosides.[112]

Shortly after the seminal discovery by Takahashi and co-workers, an *N*-acetylated version **103** was introduced by Crich et al. (Figure 8).[107c] The additional *N*-acetyl group not only effected the superb α selectivity and the yield of glycosylation,[110b] but also allowed milder basic conditions to be used for the removal of oxazolidinone group; thus, the desired *N*-acetamido product can be easily obtained. They also found the significance of nitrile solvent for the glycosylation of sterically hindered secondary acceptors, such as the 3-OH group of galactosides. The use of the more reactive donor **104** (Figure 8), which had an electron-donating admantanyl group as the leaving group, in $MeCN/CH_2Cl_2$ (1:2) at −78 °C greatly increased the α selectivity compared to the glycosylation with donor **103**.[107b]

The basic principle of the programmable one-pot approach is to assemble oligosaccharides from the nonreducing to the reducing end, with the most reactive building block being added first. Thus, since sialic acids are often found at the



Scheme 22. Synthesis of α(2→8)-linked tetrasialic acid using 5,*N*-4,*O*-oxazolidinone-protected thiosialoside donors and acceptors. Conditions: a) NIS, TfOH, M.S. 3 Å, CH_2Cl_2, −78 °C; b) thiourea, 2,6-lutidine, DMF, 70 °C; c) LiOH·H_2O, H_2O, EtOH, 80 °C, 88%; d) Ac_2O, $NaHCO_3$, H_2O, 0 °C then NaOMe, MeOH, 64%; e) $Pd(OH)_2$, H_2 (1 atm), MeOH, H_2O, 70%.

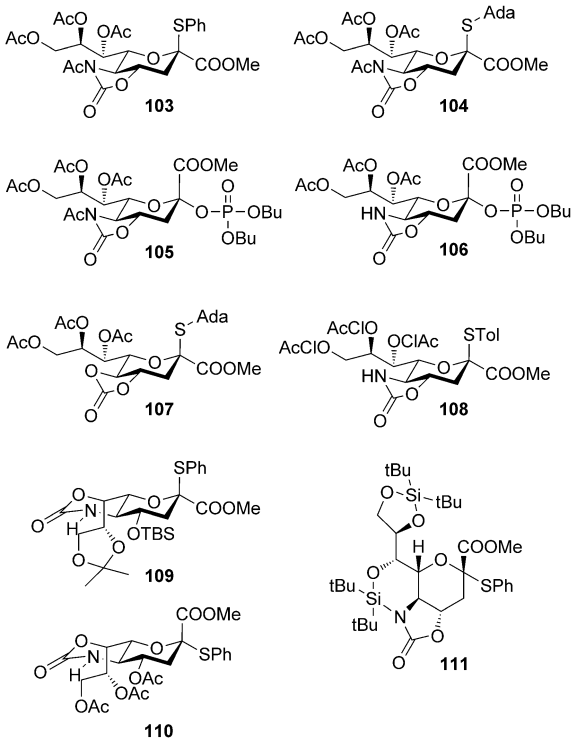
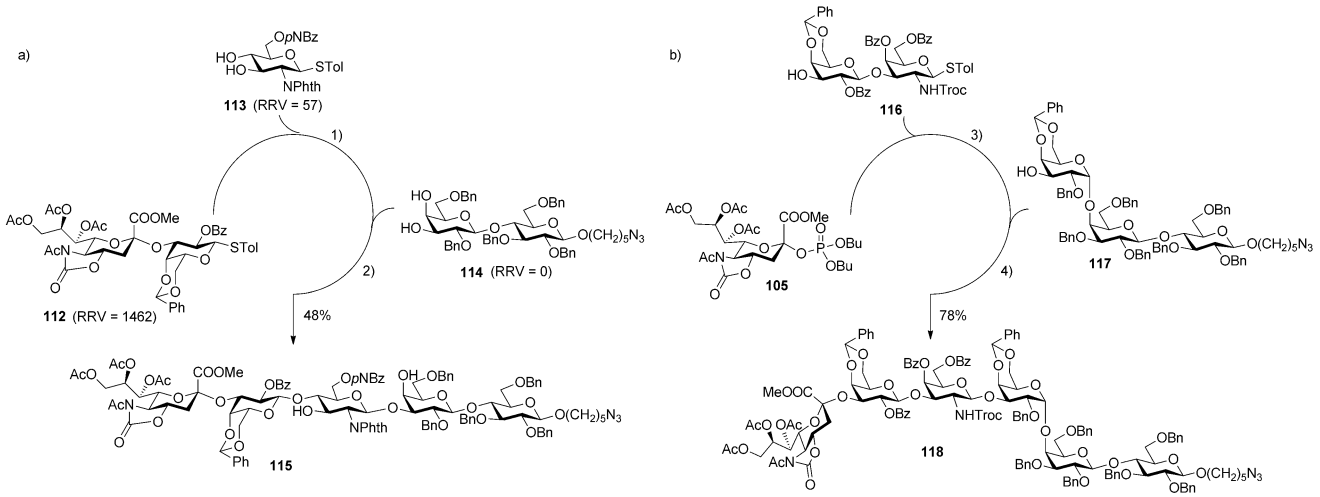


Figure 8. Recent 5-*N*,4-*O*-carbonyl-, 5-*O*,4-*O*-carbonyl-, and 5-*N*,7-*O*-protected sialyl donors.

nonreducing end, the synthesis of sialosides by this one-pot protocol should be conducted at the early stage. However, sialic acid thioglycosides are much less reactive and less influenced by the protecting groups than other glycosides.[84] For example, the calculated RRVs of thiosialosides, including two C5-modified (azido and *N*,*N*-diacetyl) donors, are less than 20, and these RRVs only increased by a limited extent even with per-*O*-benzylation (Table 1). One possible solution is to employ the sialylated thioglycosides, wherein the challenging α-sialosyl linkage is installed during the synthesis of the building blocks. This strategy requires a stereoselective sialyl donor that possesses a leaving group orthogonal to thioglycosides to prepare sialyloligosaccharide building blocks efficiently. However, an early attempt in which dibenzyl sialyl phosphite donors are used does not provide a satisfactory glycosylation.[113]

Less attention has been given to the sialyl phosphate donor because of its poor yield and poor α selectivity in glycosylation reactions.[87b,88a] Inspired by the aforementioned excellent α selectivity of oxazolidinone-protected Neu5Ac donors, Hsu et al. developed the two reactive 4,5-oxazolidinone-protected Neu5Ac donors **105** and **106** (Figure 8), bearing dibutyl phosphate as the leaving group.[89] The glycosylation of **105** with a series of thioglycoside acceptors, including at the 6-OH and 3-OH groups of galactosides and 8-OH and 9-OH groups of Neu5Ac, gave solely α-linked thiodisaccharides in high yields.[89] Without adjustment of the aglycon, the programmable one-pot synthesis of linear α(2→3)-linked sialylated pentasaccharide **115** was achieved by employing one of the thiodisaccharides, Neu5Acα(2→3)Gal **112** (RRV = 1462, Table 1), as the starting reagent (Scheme 23a).[89] The power of the phosphate donor **105** was further demonstrated by a series of one-pot reactions with an orthogonal strategy, including the first one-pot synthesis of the stage-specific embryonic antigen-4 (SSEA-4)[114] derivative **118**, in excellent yield and α selectivity (Scheme 23b).[89] Notably, these one-pot reactions can be accomplished in a short time (less than 4 h) in high yield.

More recently, Crich and Navuluri introduced the stereoselective synthesis of KDN by using a similar 4,5-*O*-carbonate-protected sialic acid donor **107** (Figure 8).[108] In this approach, the usual elimination by-products of sialylation reactions were greatly reduced. One might speculate that the increased dipole moment of the carbonate-protecting group stabilizes the positive charge of the intermediate acetonitrile adduct, thereby limiting the elimination reaction.



Scheme 23. a) Programmable one-pot synthesis using sialylated disaccharide **112**. b) Orthogonal one-pot synthesis of stage-specific embryonic antigen-4 (SSEA-4). Conditions: 1. NIS, TfOH, M.S. 4 Å, CH_2Cl_2, −78 °C; 2. NIS, TfOH, −20 °C to RT. 48% over two steps; 3. TMSOTf (1 equiv), CH_2Cl_2, −78 °C; 4. NIS, −40 °C, 78% over two steps.

The 4,5-oxazolidinone-protected sialosyl donors have great utility and potential, as evident by the highly efficient chemoselective one-pot synthesis of sialosides[89,115] and the preparation of sialyloligosaccharide building blocks with defined stereochemistry for modular synthesis,[89] an alternative strategy for synthesizing sialosides.[116] Other carbamate protecting groups of sialyl donors include 5-*N*,4-*O*-carbonyl-7,8,9-tri-*O*-chloroacetyl-protected donor **108**,[107f] 5-*N*,7-*O*-oxazinanones **109**[117] and **110**,[118] and double-locked silylene/oxazolidinone **111**[119] (Figure 8).

Overall, the programmable synthesis method provides a rapid access to oligosaccharides and an effective means to modify the glycan moiety of natural products. The building blocks used for this approach need to be optimized to be generally applicable to various structures.

4. Automated Solid-Phase Oligosaccharide Synthesis

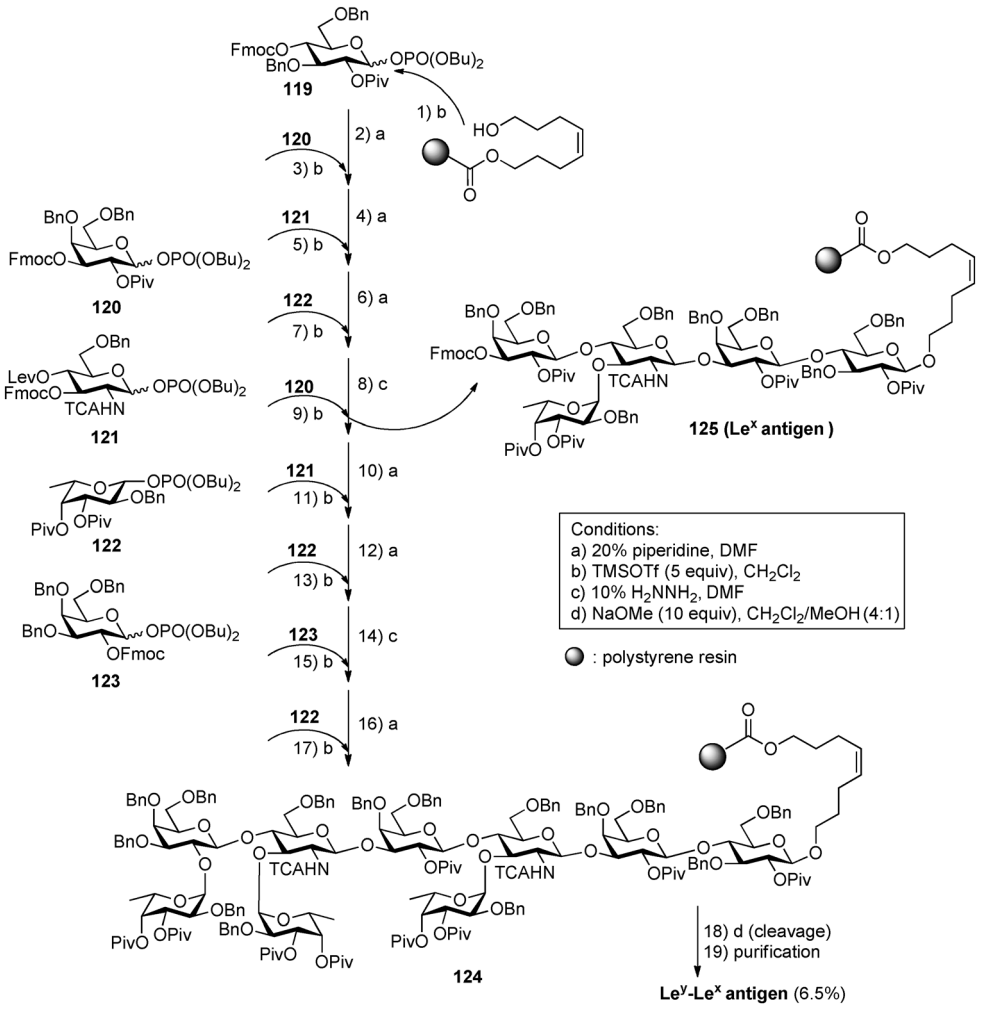
Solid-phase oligosaccharide synthesis (SPOS) offers advantages such as: 1) only one chromatography step is needed in most cases at the end of the reaction and 2) unwanted reagents and side products can be removed simply by washing and filtering, and so a large amount of the glycosyl donor can be applied to ensure the high production yield. However, the development of solid-phase oligosaccharide synthesis did not move forward much until recently. Over the past decade, advances in the glycosylation methods as well as the increased availability of protecting groups, linkers, and solid supports have driven rapid development,[120] such as the glycal-assembly-based SPOS reported by Danishefsky et al.,[121] the *O*-trichloroacetimidate-based SPOS reported by Schmidt and co-workers,[122] and thioglycoside-based SPOS by Nicolaou et al.[123] Seeberger and co-workers also introduced an automated oligosaccharide synthesizer, which was modified from an original peptide synthesizer and optimized for automated oligosaccharide synthesis (Figure 9).[124]

Figure 9. Automated oligosaccharide synthesizer developed by Seeberger and co-workers (adapted from: *Chem. Eur. J.* **2005**, *11*, 3194–3206).

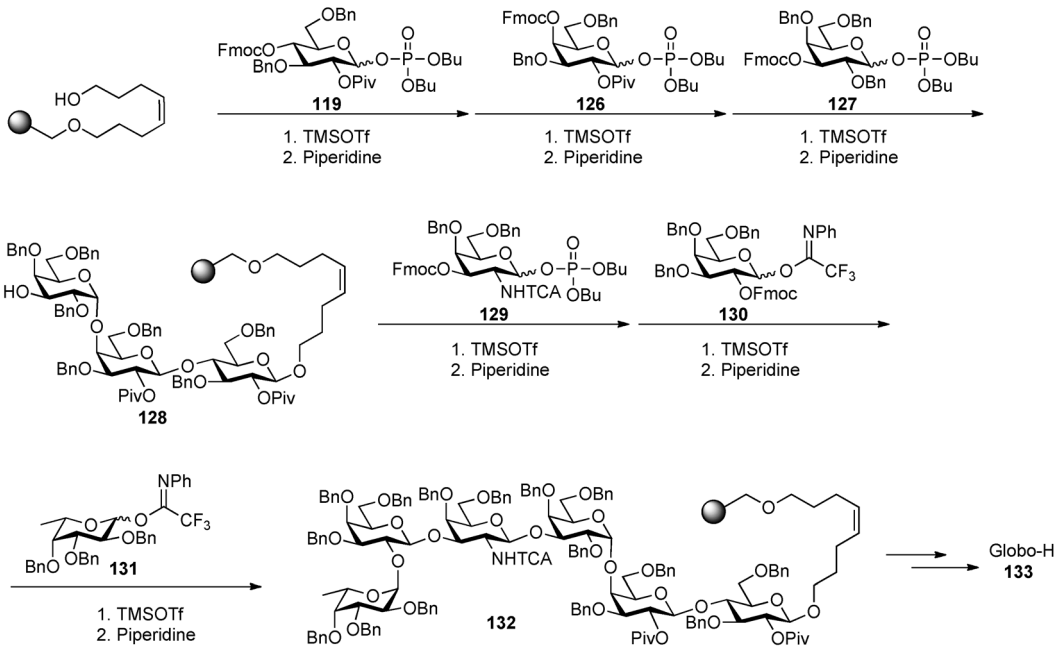
The general applicability of automated SPOS is nicely demonstrated by the recent synthesis of a nonasaccharide of Le^y-Le^x (KH-1) antigen derivative **124** (Scheme 24).[125] Glycosyl phosphates[126] **119–123** served as glycosylating agents for the construction of five different glycosidic linkages within the target KH-1 sequence. As a result of the stability of the olefinic linker to the conditions of the coupling cycle, octenediol was selected and condensed with carboxy-terminated polystyrene resin to form an ester linkage which can be rapidly cleaved with strong base at the end of the synthesis. The 9-fluorenylmethyloxycarbonyl (Fmoc) group was used as the temporary protecting group because of its excellent stability toward acidic glycosylation conditions and its ease of cleavage with non-nucleophilic amines. In addition, the dibenzofluorene generated after cleavage of the Fmoc group can be used to monitor the deprotection efficiency by UV/Vis spectroscopy. Levulinoyl ester was used as another temporary protection for the branching point and can be readily cleaved by hydrazine. In short, general automated synthesis protocols involved the following steps: 1) coupling: TMSOTf (5 equiv) for the activation of glycosyl phosphate donors was added twice to ensure a high coupling yield and to drive the coupling reaction to completion. 2) Deprotection: the temporary Fmoc protecting group was selectively removed by using 20% piperidine in DMF, and the levulinoyl ester was removed by using 10% hydrazine in DMF. 3) The target oligosaccharide product was released by using sodium methoxide; cross-metathesis with ethene in the presence of the Grubbs catalyst can also be used to generate *n*-pentenyl glycosides, which can be converted into other functional groups such as a thio group for biological purposes.[127] HPLC purification was the only purification step before global deprotection. The KH-1 (Le^Y-Le^X) nonasaccharide was synthesized in 23 h in an overall yield of 6.5%. The Le^x antigen **125** could also be synthesized during the glycan chain elongation (Scheme 24).

The automated oligosaccharide synthesis has been used to synthesize several important carbohydrates. These include globo-H hexasaccharide **133** (Scheme 25),[128] the core pentasaccharide of *N*-linked glycans,[129] β-mannoside,[130] oligomannosides,[131] oligorhamnosides,[132] the phytoalexin elicitor family of glucans,[124a] and the parasitic vaccine candidates against malaria and leishmaniasis (see Section 7).

The stereoselective installation of 1,2-*cis*-glycosides remains one of the major obstacles for SPOS, although some progress has been made for the α-galactosidic[128] (Scheme 25) and β-mannosidic[130] linkages. Recently, Boons and co-workers elegantly addressed this problem by using a chiral-auxiliary-mediated 1,2-*cis*-glycosylation.[133] They showed that a (*S*)-(phenylthiomethyl)benzyl chiral auxiliary



Scheme 24. Automated solid-phase synthesis of Le^x and Le^y-Le^x (KH-1).



Scheme 25. Automated solid-phase synthesis of globo-H hexasaccharide.

attached at the C2-position of the glycosyl donor participates in the glycosylation reaction through the formation of an anomeric equatorial sulfonium ion as a *trans*-decalin ring which is subsequently replaced by a sugar alcohol to synthesize a 1,2-*cis*-glycoside stereoselectively (Figure 10).[134] By using this method, a series of branched oligoglucosides containing multiple 1,2-*cis*-glycosidic linkages were successfully assembled on the solid support with high stereoseletive control and high yields (Scheme 26).[133] The (*S*)-(phenylthiomethyl)benzyl chiral auxiliary can be easily converted into an acyl group on the solid support and is compatible with the conditions for the selective removal of the temporary protecting groups Fmoc and Alloc.

Despite the successes in automated solid-phase oligosaccharide synthesis, the need for large excesses of sugar donors (e.g. 10 equivalents for each coupling step) to force the reaction to completion still necessitates additional steps for the preparation of building blocks. Alternatively, the light fluorous tag assisted solution-phase method, in which the desired tagged compound can be separated from the reaction mixture by fluorous solid-phase extraction[135] (FSPE) through specific solvophobic interactions, has emerged as an attractive strategy for the automated synthesis of carbohydrates.[136] The light fluorous tag (C_8F_{17}) has been demon-

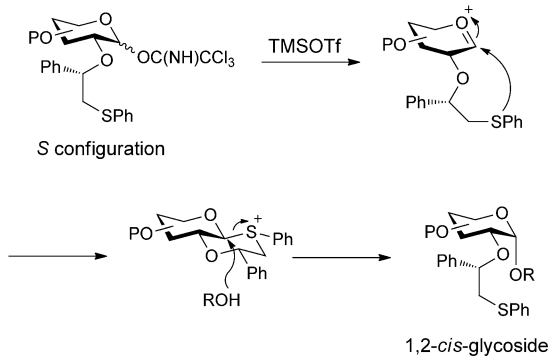
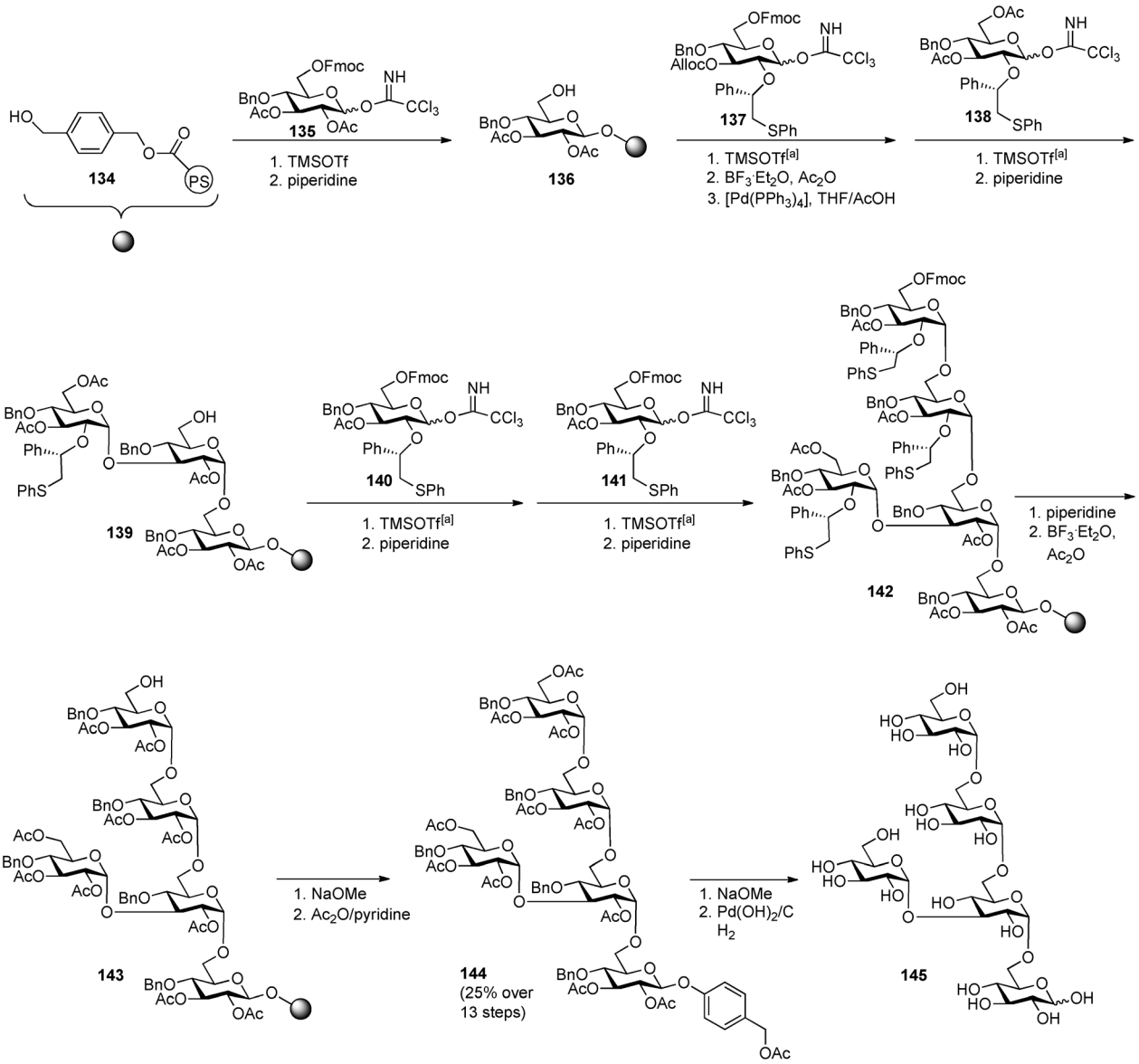
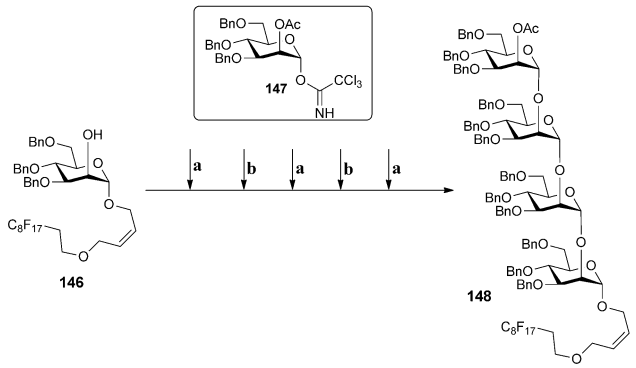


Figure 10. Installation of a 1,2-*cis*-glycoside mediated by a chiral auxiliary.

strated to be inert to typical conditions for glycosylation and protecting-group manipulation in carbohydrate chemistry, and offers the ease of characterization of carbohydrate intermediates by NMR spectroscopy.[136,137] Recently, Jaipuri and Pohl employed this approach to synthesize tetra- and pentamannose oligosaccharides by using a fluorous tag incorporated within an oxygen spacer (Scheme 27),[136] which can significantly increase the solubility of the oligosaccharides and the linker itself in organic solvents commonly used in the glycosylation reaction. By iterative coupling, filtering, and deprotection, an α(1→2)-linked tetramannoside **148** can be synthesized in 79% overall yield (Scheme 27). Only six equivalents of **147** was required, which is much less than that required for the automated solid-phase method.[124a] This fluorous-tag method is also applicable to the synthesis of a branched mannose pentasaccharide.[136] Levulinic ester and/



Scheme 26. Stereoselective solid-phase synthesis of α(1→6)-linked tetraglucoside branched with an α(1→3)-linked glucose. [a] The imidate donors **137**, **138**, **140**, and **141** were preactivated by TMSOTf in −40 °C in a separate reaction flask.



Scheme 27. Iterative synthesis of α(1→2)-linked tetramannoside. Reaction conditions: a) 1. Coupling (**147**, TMSOTf, CH_2Cl_2); 2. filtration (FSPE); b) 1. deprotection (NaOMe, MeOH); 2. filtration (FSPE), 79% overall yield.

or TBDPS ether were/was employed as temporary protecting group(s) for the branching points. Notably, these branched or linear mannose structures were highly soluble in the aqueous organic mixtures used for loading the FSPE columns.

This fluorous tag is also of interest as it has an alkene group that can be transformed into other functional groups. In this way, synthetic oligosaccharides can be conjugated into multivalent structures.[138] Alternatively, sugars tagged with a single C_8F_{17} tag can be noncovalently attached to perfluorinated glass slides, which can be used for carbohydrate microarrays and screening against carbohydrate-binding proteins.[139] Very recently, the fluorous tag was also applied to the one-pot oligosaccharide synthesis to facilitate the purification of desired oligosaccharides.[140]

While SPOS holds great potential for the synthesis of oligosaccharides, it has its limitations, especially in regard to the problems of protecting-group manipulation, the need for large excesses of glycosylation reagents, and the creation of molecular diversity. The complexity of selective deprotection increases as the chain length increases. In addition, it is difficult to rapidly create molecular diversification with the method, although it would speed up the synthesis of the target glycan and simplify the intermediate purification process. Further improvements are needed to make solid-phase oligosaccharide syntheses more user friendly: 1) precise control of the stereoselectivity and regioselectivity, and 2) quantitative glycosylation in every coupling step. Otherwise, the increasing number of mixed α/β linkages within the growing oligosaccharide chain and deletion sequences will greatly slow down the final purification step.

4.2. Solid-Phase Glycopeptide Synthesis

Among posttranslational modifications, glycosylation is a common and complex modification that is estimated to be present in more than 50% of all the human proteins. The glycosylation of proteins usually occurs at Ser, Thr, and Tyr (*O* glycosides) or at Asn (*N* glycosides), and leads to a wide range of biological functions.[1c] Access to homogeneous glycoproteins and glycopeptides is of vital importance for understanding the role of glycosylation at the molecular level. As a consequence of the widespread existence of heterogeneous glycoforms, which result in an availability problem of homogeneous forms, chemical and chemoenzymatic synthesis have become efficient and reliable approaches to acquire essential glycopeptides and glycoproteins for biological and structural studies.[141] Three common strategies have been frequently employed to obtain glycopeptides by solid-phase synthesis (Figure 11):[142]

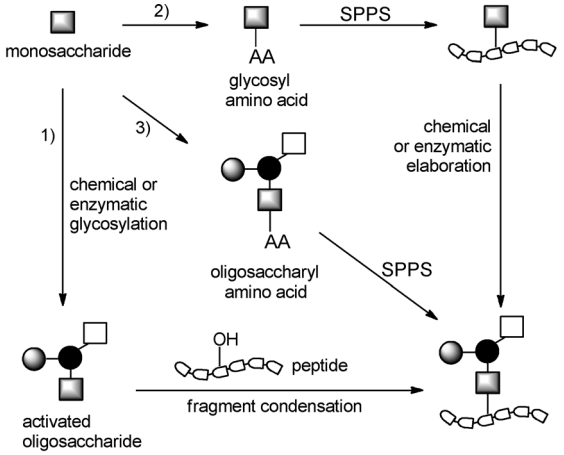


Figure 11. Three strategies to prepare glycopeptides with complex glycans.

1. Convergent fragment condensation of activated oligosaccharides with properly protected full-length peptides;
2. Stepwise solid-phase peptide synthesis of a simple glycopeptide, followed by elongation of the glycan;
3. Stepwise solid-phase peptide synthesis by using fully glycosylated amino acid building blocks.

The direct condensation of the saccharide and peptide fragments represents a highly convergent approach to the synthesis of glycopeptides. The complex glycodomain could be built first, such as by the one-pot or solid-phase strategy outlined above, and then the ultimate product with an activated anomeric position could be directly condensed with the appropriately protected peptide. Direct *O*-glycosylation of the free hydroxy groups of Thr or Ser in peptides on a solid phase is possible,[143] although it is still hampered by the poor solubility of the peptides under common glycosylation conditions, which results in low yield. In the case of *N*-glycosylated peptides, the reaction of a glycosylamine with the aspartic side chain is a favored approach. The power of this method was elegantly exemplified by the syntheses of *N*-glycopeptides bearing all types of complex *N*-glycans, including high-mannose-type,[144] hybrid-type,[145] and complex-type by Danishefsky and co-workers.[146] In their approach, the glycosylamine carrying the complex *N*-glycans were typically synthesized by using the glycal assembly system followed by transformation of the reducing sugars to glycosylamines by using the Kochetkov reaction.[147] Although useful, the limitation of this method includes the anomerization of the glycosylamine under reduction conditions, and the formation of intramolecular aspartimides during the aminolysis reac-

tion. There is still a need for alternative strategies to obtain *N*-glycosyl amides,[148] such as the use of glycosylazides for the stereoselective *N*-glycosylation by a Staudinger ligation.[149]

Most commonly, preformed glycosyl amino acid building blocks bearing either monosaccharide or oligosaccharide side chains are employed in the stepwise solid-phase peptide synthesis (SPPS). The synthetic route to the saccharide components and the glycosylation reactions to furnish the *O*- and *N*-glycosyl amino acid building blocks have been reviewed extensively elsewhere.[150] The incorporation of glycosyl amino acid bearing bulky saccharide components in SPPS often results in a low-yielding coupling. Therefore, it is sometimes more strategic to use a glycosyl amino acid containing simple glycans in the peptide assembly and then elaborate the glycopeptides enzymatically. As such, the simple *O*- or *N*-linked glycopeptides can either function as acceptors for the stepwise addition of a monosaccharide by specific glycosyltransferases, such as the synthesis of *O*-glycopeptide fragments of the cell adhesion molecule PSGL-1 (P-selectin ligand-1),[151] or for the transglycosylation wherein one enzyme such as *endo*-β-*N*-acetylglucosaminidase (ENGase) transfers a fully assembled oligosaccharide in a single step.[152] The availability problem of glycosyl amino acids carrying large and complex glycans can also be solved by direct isolation from natural glycoproteins. Several examples which incorporated nature-derived oligosaccharyl amino acids into SPPS have been demonstrated to be useful for the synthesis of *N*-glycopeptides.[153]

Although solid-phase synthesis allows the automation of the highly repetitive process of coupling building blocks, the routine synthesis of peptides of greater than 50 amino acids is limited. However, it is possible to use glycopeptide segments for further ligation reactions to extend short glycosylated peptides to glycoproteins.[154]

5. Efficient Strategies Expediting Enzymatic Oligosaccharide Synthesis

Enzymes have great potential as catalysts for use in a variety of synthetic problems.[3h,155] For example, enzyme-catalyzed glycosylation features exquisite regio- and stereoselectivity under very mild conditions, and extensive protecting-group manipulation is unnecessary.[156] This section will focus mainly on strategies that expedite enzyme-catalyzed oligosaccharide synthesis. Recent reviews are recommended for further information on this subject.[70,156c,157]

5.1. Regeneration of Sugar Nucleotides

The glycosyltransferases,[158] which catalyze the transfer of a saccharide from a sugar nucleotide donor to an acceptor, have been used for the synthesis of complex glycoconjugates.[156c,157b,d,159] Nevertheless, restricted substrate specificity, high cost of both the enzymes and the sugar nucleotides, and limited enzyme availability narrow their application. Moreover, the progression of glycosylation can be plagued by feedback inhibition caused by the generated nucleoside phosphate. Regeneration of the sugar nucleotide intermediate in situ can reduce the concentration of the nucleoside phosphate by-product by the use of less than a stoichiometric amount of the sugar nucleotide; thus, the expense of the sugar nucleotide and product inhibition are reduced simultaneously.[160] Many glycosyltransferases involved in the biosynthesis of oligosaccharides utilize only nine sugar nucleotides as donor substrates in mammalian systems, including α-UDP-glucose (UDP-Glc), α-UDP-*N*-acetylgalactosamine (UDP-GalNAc), α-UDP-glucuronic acid (UDP-GlcA), α-UDP-*N*-acetylglucosamine (UDP-GlcNAc), α-UDP-galactose (UDP-Gal), α-UDP-xylose (UDP-Xyl), β-GDP-fucose (GDP-Fuc), α-GDP-mannose (GDP-Man), and β-CMP-*N*-acetylneuraminic acid (CMP-Neu5Ac).

Almost three decades ago, Whitesides and co-workers first reported a multienzyme system for the large-scale synthesis of *N*-acetyllactosamine (LacNAc) with the in situ regeneration of UDP-Gal (Figure 12a).[161] This multienzyme system integrated the recycling of UDP-Glc used to generate UDP-Gal by UDP-Gal 4-epimerase. Subsequent consumption of the UDP-Gal by β1→4GalT gave LacNAc on a multigram scale. Over the last two decades, efforts have been made to develop regeneration systems for all common sugar nucleotides.[156c,d,162] For example, in addition to the above-mentioned recycling system, UDP-Gal can also be regenerated either by employing Gal-1-phosphate (Gal-1-P) from galactose by galactokinases,[163] or by incorporating sucrose synthetase into the recycling scheme.[164] Usually it is better to use a kinase to make a sugar-1-phosphate, which then reacts with UTP to form the UDP-sugar. For the sialyltransferase, CMP-Neu5Ac can be simply regenerated from Neu5Ac.[165] To reduce the cost, an alternative, cheaper starting material is ManNAc, which can be converted into Neu5Ac with a Neu5Ac aldolase catalyst (Figure 12b).[166]

Glycosyltransferase-based systems in which the regeneration of sugar nucleotides is achieved have been reported for the synthesis of complex oligosaccharides.[156c,157e,162a] The application of UDP-Gal regeneration in combination with various galactosyltransferases has led to the syntheses of α-Gal epitopes[164,167] and LacNAc.[161,163,168] A multienzyme regeneration system for UDP-GalNAc that functioned with β1,3-*N*-acetylgalactosaminyltransferase from *Haemophilus influenza* has been utilized for the large-scale synthesis of important globotetraose and isoglobotetraose.[169] The hyaluronic acid (HA) polymer could be synthesized efficiently by a recombinant HA synthase with in situ regeneration systems of UDP-GlcA along with UDP-GlcNAc.[170] Notably, methods employing the combined sequential use of glycosyltransferases together with the regeneration of the nucleotide sugar have also been investigated for the synthesis of complex oligosaccharides such as sialyl Lewis x (sLe^x), with in situ regeneration of CMP-Neu5Ac, UDP-GlcNAc, UDP-Gal, and GDP-Fuc.[168a]

Phosphoenolpyruvate (PEP)[155] is frequently used in sugar nucleotide regeneration as a phosphate source because of its stability to hydrolysis and its strong phosphorylating ability. However, several other systems have also been investigated. Wang and co-workers recently reported the use of the creatine phosphate-creatine kinase (CP-CK) as an

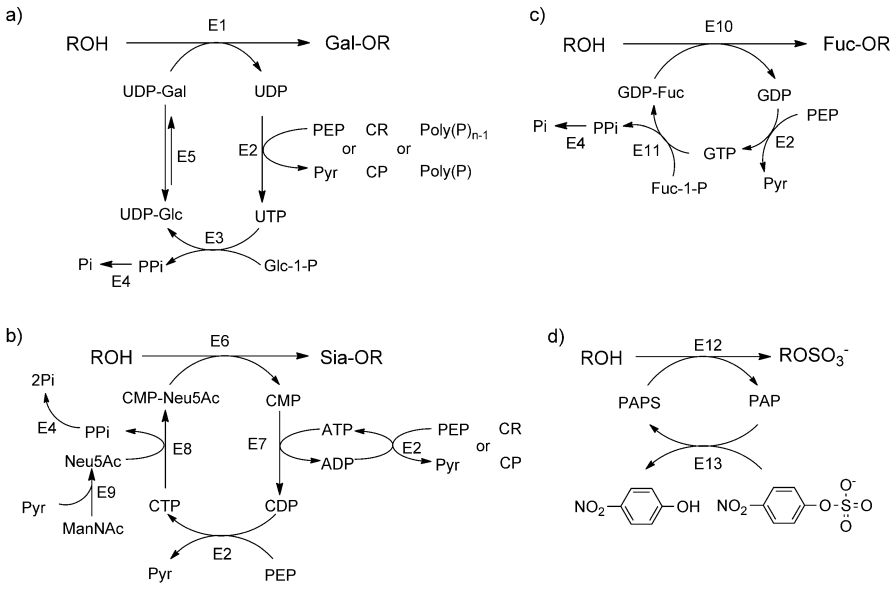


Figure 12. Recycling system for the regeneration of a) UDP-Gal, b) CMP-Neu5Ac, c) GDP-Fuc, and d) PAPS. E1 = galactosyltransferase, E2 = pyruvate kinase (PK) or creatine kinase (CK) or *E. coli* polyphosphate kinase, E3 = glucocose-1-phosphate uridylyltransferase, E4 = pyrophosphatase (PPase), E5 = UDP-Gal 4-epimerase, E6 = sialyltransferase, E7 = nucleoside monophosphate kinase (NMK), E8 = CMP-Neu5Ac synthetase, E9 = Neu5Ac aldolase, E10 = fucosyltransferase, E11 = GDP-Fuc pyrophosphorylase, E12 = sulfotransferase, and E13 = aryl sulfotransferase. PEP = phosphoenolpyruvate, Pyr = pyrate, CP = creatine phosphate, CR = creatine, PAPS = phosphoadenosyl phosphosulfate, PAP = phosphoadenosyl phosphate, PPi = inorganic pyrophosphate.

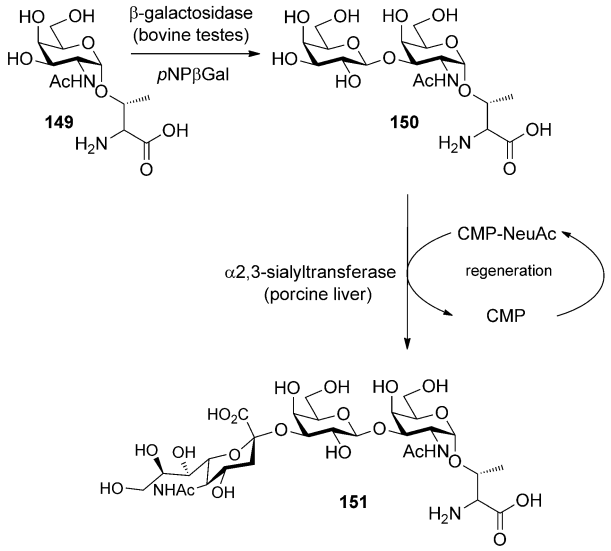
alternative energy supply system for the synthesis of oligosaccharides.[171] Since the standard free energy of hydrolysis of CP ($-43.1\ kJ\,mol^{-1}$) is higher than that of ATP ($-30.6\ kJ\,mol^{-1}$), CDP or ADP is readily phosphorylated into their respective triphosphates by the CP-CK system. Coupling this system to the known regeneration systems, such as UDP-Gal and CMP-Neu5Ac (Figure 12a,b) gave several oligosaccharides, for example, globotriose with yields comparable to the use of the PEP-PK (pyruvate kinase) system.[171] The use of polyphosphate kinase from *E. coli* together with poly(P) has also been applied as an economical nucleoside triphosphate regeneration system of UDP-Gal, which was successfully employed in the synthesis of LacNAc.[172]

The regeneration of phosphoadenosyl phosphosulfate (PAPS) from its by-product phosphoadenosyl phosphate (PAP) by using aryl sulfotransferase was also developed for the synthesis of sulfated glycans and glycopeptides (Figure 12d).[68]

5.1.1. *One-Pot Multiple Enzyme Synthesis with In Situ Sugar Nucleotide Regeneration*

Since the glycosyltransferases can be widely used in reaction sequences with other glycosyltransferases or enzymes such as glycosidases, sulfotransferases, and proteases,[156c] it is convenient to transform complicated multiple-enzyme-catalyzed reactions into one-pot reactions, especially those based on glycosyltransferases coupled with sugar nucleotide regeneration. This strategy has been used in the one-pot multienzyme-catalyzed synthesis of 6′-sialyllactosamine with in situ regeneration of CMP-NeuAc and UDP-Gal,[166] and the synthesis of Galα1→3Galβ1→4GlcNAcβ1-OR trisaccharide with β1→3GalT and β1→4GalT in the presence of the UDP-Gal recycling system.[164]

Glycosidase-catalyzed transglycosylation in combination with the glycosyltransferase-catalyzed system and the regeneration of sugar nucleotides has also been utilized in one-pot syntheses.[173] In an elegant example, Křen and Thiem employed β-glycosidase to form TF (Galβ1→3GalNAcα1→OThr) antigen disaccharide **150** from GalNAcα1→OThr **149**, which was immediately treated with α2→3-sialyltransferase to give sialyl TF antigen **151** with in situ regeneration of CMP-Neu5Ac (Scheme 28).[174] In this case, the hydrolysis of the intermediate disaccharide by the glycosidase can be blocked by its glycosyltransferase-mediated conversion into a nonhydrolyzable product.



Scheme 28. One-pot multiple enzyme synthesis of sialyl-TF antigen by using a mixed catalytic system.

5.1.2. *Oligosaccharide Synthesis with Sugar Nucleotide Regeneration Beads or Engineered Whole Cells*

Although the coupling of glycosyltransferases-based syntheses with in situ sugar nucleotide regeneration has been

proved to be very useful, a primary disadvantage is the requirement of purifying the multiple enzymes involved in the synthesis, which can be time consuming.

To avoid this, Wang and co-workers recently introduced "superbead" technology, wherein recombinant His_6-tagged enzymes required for the regeneration of UDP-Gal were all immobilized on nickel nitrilotriacetate agarose beads.[175] These beads were used as a normal reagent in combination with various galactosyltransferases, including α1→3GalT, α1→4GalT, and β1→4GalT, to synthesize oligosaccharides such as globotriose (Gb3) and isoglobotriose (iGb3), and could be reused several times with only a slight decline in enzyme activity. A simpler three-enzyme UDP-Gal regeneration bead that used commercially inexpensive UTP has recently been utilized to synthesize Gb3 and iGb3.[176] The shortcoming of this approach is that when one or two of the immobilized enzymes are inactivated, detection and replacement may be problematic.

Alternatively, by using genetic engineering, one can incorporate all the genes for the enzymatic synthesis of an oligosaccharide into the bacteria to produce the product on large scales in a fermentation or cell-culture system, with no need to isolate the enzymes.[177] With this strategy, Koizumi et al. at Kyowa Hakko Kogyo Co. developed a system for the large-scale production of oligosaccharides by coupling genetically engineered bacteria.[178] Starting from a key technology wherein a *Corynebacterium ammoniagenes* bacteria produced a high level of UTP from inexpensive orotic acid, the combination of *Escherichia coli* cells engineered to overexpress genes and the biosynthetic pathway produced a large quantity of sugar nucleotides and oligosaccharides. This innovative technology has been applied to the multigram synthesis of oligosaccharides including globotriose,[178] 3′-sialyllactose,[179] and several sugar nucleotides such as UDP-Gal,[178] GDP-Fuc,[180] and CMP-Neu5 Ac[179].

An attractive alternative to the aforementioned strategies is to utilize a living factory approach, a method in which oligosaccharides are synthesized in a single living bacterium that was simply engineered to express the required glycosyltransferases and to possess its cellular machinery required for the production of sugar nucleotides.[181] By using this strategy, several elegant examples of the gram-scale production of oligosaccharides, such as sialylated oligosaccharide (GM2[182] and GD3[183]) and fucosylated oligosaccharides (Le^x,[181c] H antigen[181b]), were reported.

5.2. Enzymatic Oligosaccharide Synthesis on a Solid Phase or Polymer Support

Similar to the automated solid-phase synthesis of oligosaccharides, it is also desirable that the enzymatic synthesis is carried out in combination with a solid phase. In this respect, the approach could offer a real simplification by combining the advantages of the enzymatic approach with those of the solid-phase method; that is, easy purification of the product with stereo- and regiocontrol as well as the elimination of intermediate protecting-group manipulations.[184] Incorporating the enzyme into the solid-phase strategy has resulted in advantages, such as an increased stability toward denaturation, the ease of product separation, and enzyme recovery. Fixing the reaction partners on the solid phase can be achieved in a linear fashion from the reducing end by approaches in which either the acceptor saccharide or enzyme is attached to the solid support. The key issue in the solid-phase strategy is to find an appropriate solid support, which should be hydrophilic to allow good swelling in water, and suitable for use in organic solvents such as DMF, or rigid so the enzyme will not become entrapped. The length of the linker employed to connect the acceptor saccharide to the solid support is also crucial.[185] A longer linker may increase the conformational flexibility and reduce the steric interference between the enzyme and the solid phase, thus making the acceptor sites more accessible to the enzymes. Many resins have been used, including controlled pore glass (CPG) support,[186] derivatized silica,[187] sepharose,[185] and polyethylene glycol polyacrylamide polymer (PEGA).[188] The covalent immobilization of glycosyltransferase on sepharose beads has also been demonstrated to be successful for an enzyme transfer reaction.[189]

Water-soluble polymer supports, which combine the advantages of the solution-phase regime with ease of product recovery, have also been used for the enzymatic synthesis of oligosaccharides. It has been demonstrated that immobilization of the sugar acceptor on the polymer support may result in a "polymeric sugar-cluster effect" which enhances the affinity of sugar substrates for the glycosyltransferase and thus improves the glycosylation.[190] Furthermore, the glycosylation could be monitored and analyzed by NMR and fluorescence spectroscopy. Nishimura and co-workers have made great contributions to the use of polyacrylamide as the water-soluble polymer for the enzymatic synthesis of sialyl *N*-acetyllactosamine,[189–190,191] sLe^x,[190b] and ganglioside GM3.[192] They also developed an efficient enzyme recycling strategy, whereby the glycosyltransferase was produced as a fusion protein with maltose-binding protein, which acts as an affinity tag for the immobilization of these engineered biocatalysts to the solid support such as amylose resin.[190b] The combined use of such an immobilized glycosyltransferase and water-soluble polymers as the acceptor substrates has proved to be successful in the glycosynthetic system.[189,190b] A prototype "automated glycosynthesizer" was developed according to this strategy (Figure 13).[193] Other water-soluble polymers such as thermoresponsive polyacryamide has also been described for the synthesis of the sLe^x tetrasaccharide,[194] where the polymer carrying the acceptor was soluble at low temperature and insoluble at high temperature.

The strategy of using a combination of solid-phase peptide synthesis and solution-phase enzymatic synthesis has been used successfully in the preparation of glycoconjugates such as glycopeptides. A glycosyl amino acid building block was incorporated in a usual way into the solid-phase peptide synthetic scheme to give a glycopeptide which then served as a primer for subsequent enzymatic elaboration. For example, PSGL-1 glycopeptides required for the high-affinity binding reaction of P-selectin,[151a,b,195] and *O*-glycopeptides of the mucin domain of L-selectin ligand MAdCAm-1,[188b] have been efficiently synthesized by this approach. The strategy

Figure 13. Automated glycosynthesizer (adapted from Ref. [193]).

was recently extended by Nishimura and co-workers, who designed the molecular transporter which was used to blot the glycopeptides released from the solid-phase into the water-soluble polymer for further enzymatic elongation.[196] The transporter is actually a multifunctional linker which either served as a tag to attach the glycopeptide onto the polymer or as a cleavage site to facilitate release of the product from the polymer. This technique was applicable to the combinatorial synthesis of a MUC1 glycopeptide library containing 36 glycoforms.[196b]

Although promising, the number of enzymes available for solid-phase synthesis is still very limited, and further efforts are needed to increase the number.

5.3. Chemoenzymatic Oligosaccahride Synthesis

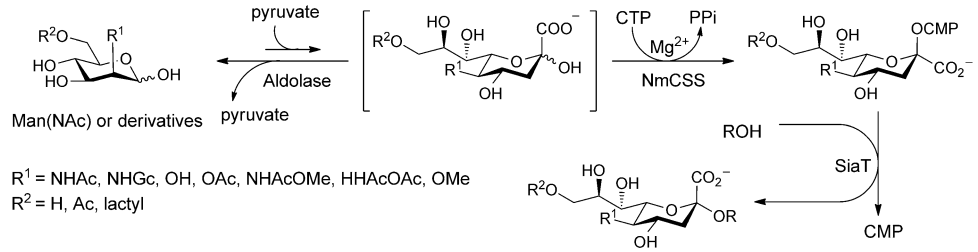
Chemoenzymatic synthesis, which combines the regio- and stereoselectivity of enzymes with the flexibility of chemical synthesis, has recently emerged as a potential method for carbohydrate synthesis.[3h,156,157b,159a,b,184,197] Enzymes such as glycosyltransferases or glycosidases can be employed to append saccharide moiety from a suitable donor to the chemically derived oligosaccharides or glycoconjugates.[152b,198] As a result of their strict regiospecificity and stereospecificity, they are particularly useful for the formation of glycosidic bonds which are difficult to accomplish otherwise, such as the synthesis of α-sialosides. To tackle this problem chemically, strategies (e.g. sialylated oligosacchariade building blocks) have to be developed, but they will certainly increase the complexity of the building block design. The reaction sequence in the chemoenzymatic oligosaccharide synthesis can also be reversed, so products from enzymic synthesis can be used for chemical elaboration.[176,199] These two types of reaction sequences can be tailored to accommodate the most suitable combination for the derivatization of specific glycosylated compounds.

5.3.1. Chemoenzymatic Oligosaccharide Synthesis by Glycosyltransferases

Glycosyltransferases from bacteria were thought to be more suitable for chemoenzymatic oligosaccharide synthesis, as their mammalian counterparts are in general less "promiscuous" and are difficult to express in *Escherichia coli*, and therefore limited in their availability for large-scale synthesis.[177c,200] Recently, the expanding number of cloned bacterial glycosyltransferases[201] with wide substrate specificity have led to the parallel and combinatorial chemoenzymatic syntheses of various natural and non-natural oligosaccharides.

Chen and co-workers have recently developed a one-pot multienzyme system for the efficient synthesis of sialosides (Table 4).[85a,202] In this system, recombinant K-12 sialic acid aldolase from *E. coli* catalyzed the synthesis of sialic acid precursors for CMP-Neu5Ac from their corresponding hexose derivatives (mannose, *N*-acetylmannosamine, and other derivatives). CMP-Neu5Ac derivatives were then generated in situ from sialic acid precursors catalyzed by a recombinant CMP-sialic acid synthetase NmCSS and subsequently used by sialyltransferase to transfer the sialic acid residue to a sugar acceptor for the formation of natural sialosides. These three steps can be combined in a one-pot sequence without purification of the intermediates. This approach has been demonstrated to be highly efficient for the systematic synthesis of highly diverse sialoside libraries. A number of natural and non-natural sialoside derivatives containing α(2→3),[202a] α(2→6),[203] and α(2→8)[204] linkages were synthesized in large quantities and in high yields with versatile structural modifications, mainly at the C5- and C9-positions of the sialic acid residues. It is also of great importance that 3-fluoro-Neu5Ac- and Neu5Gc-containing sialyllactose trisaccharides, which are particularly difficult to obtain by organic synthesis, could be prepared by using

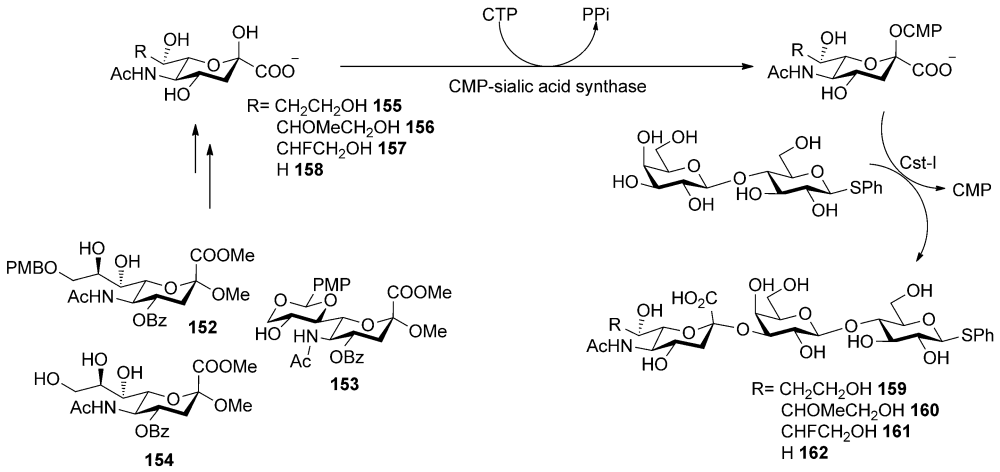
Table 4: One-pot multienzyme approach according to Chen and co-workers for the synthesis of sialosides with versatile structural modification.



	Enzyme	Source	Function
SiaT	aldolase[207]	*E. coli* K-12	sialic acid aldolase
	NmCSS[207]	*Neisseria meningitidis*	CMP-sialic acid synthetase
	Pm0188Ph(PmST1)[202a]	*Pasteurella multocida*	α(2→3)-sialyltransferase
	Pd2,6ST[203]	*Photobacterium damsela*	α(2→6)-sialyltransferase
	CstIIΔ32^{153S}[204,208]	*Campylobacter jejuni*	α(2→8)-sialyltransferase

NmCSS and multifunctional sialyltransferase PmST1 in a one-pot two-enzyme synthesis.[205] The 3-fluorinated sialic acid derivatives have been demonstrated to be competitive inhibitors for virus sialidases.[206]

A strategy of chemoenzymatic synthesis of sialic acid analogues modified at position 8 was recently reported by Morley and Withers (Scheme 29).[209] The modified sialic acid precursors **155**–**158** were synthesized from compounds **152**–**154**, and each was converted into its cytidine monophosphate (CMP) donor by using a bacterial CMP-sialic acid synthetase. Cst-1, an α2,3-sialylatransferase from *Campylobacter jejuni*, was successfully applied to the synthesis of sialyl thiolactosides **159**–**162**. Notably, a comparison of the hydrolysis activity between Cst-1 and another sialyltransferase, PM0188h, revealed that Cst-1 is preferential for the synthesis of 8″-modified sialyllactose, while PM0188h exhibited more hydrolysis activity toward natural substrates.[209]

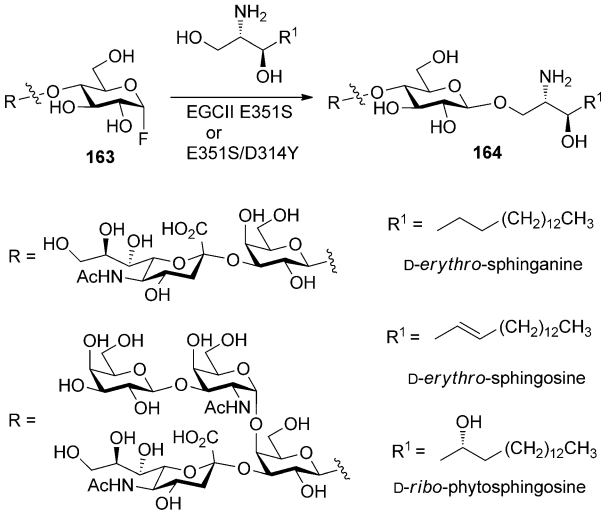


Scheme 29. Chemoenzymatic synthesis of sialyl lactose trisaccharide with C-8 modifications.

In another study by Chen and co-workers, two bacterial β-1,4-galactosytransferases, NmLgtB and Hp1-4GalT, showed promiscuous and complementary specificity for GlcNAc monosaccharide substrates with 6-*O*- or *N*-sulfate groups.[210] The use of these enzymes in the one-pot multienzyme synthesis led to the efficient generation of an array of LacNAc and lactose derivatives.[210] The chemoenzymatic synthesis of sulfur-linked saccharides was also investigated.[211] For example, Withers and co-workers recently reported an efficient synthesis of the sulfur-linked disaccharides Gal-β-S-1,4-GlcNAc*p*NP and uncommon Gal-β-1,4-Man-*p*NP by using the β1,4-galactosyltransferase HP0826 from *Helicobacter pylori*.[212]

5.3.2. *Chemoenzymatic Oligosaccharide Synthesis by Glycosidases and Glycosynthases*

Glycosidase-catalyzed transglycosylation has some advantages over glycosyltransferases, such as the use of readily available donor substrates, the easy availability of enzymes, and the potential for block transfer of oligosaccharides. Nevertheless, although some glycosidases have become valuable biocatalysts for building regio- and stereospecific glycosidic linkages in saccharide structures,[157a,c,159b–d] two major limitations, including low glycosylation yields and product hydrolysis, are frequently encountered. These problems can be overcome by mutation of the nucleophile residues in the catalytic center to generate mutant glycosidases, namely, glycosynthases, which are devoid of hydrolase activity with retention of their glycosylation activity. Further improvement can be obtained through directed evolution.[157a,213] For example, an efficient chemoenzymatic synthesis of *lyso*-GM1 and *lyso*-GM3 antigens was achieved with a glycosynthase (E351S) evolved from the endoglycoceramidase II (EGC II), which was able to use synthetic glycosyl fluoride **163** as a donor sugar to glycosylate sphinganine and sphingosine derivatives in high yields (Scheme 30).[214] However,

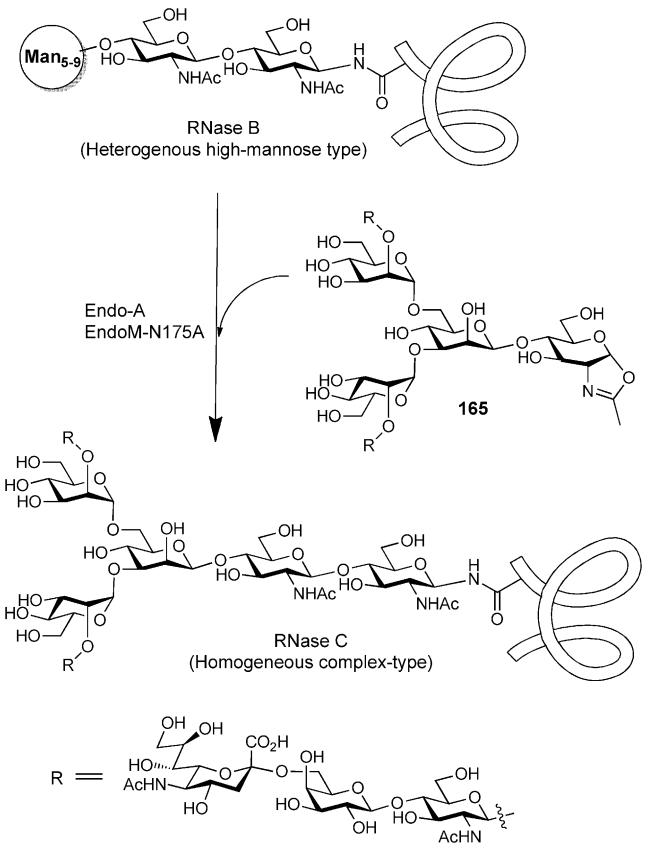


Scheme 30. Synthesis of glycosphingolipid derivatives by EGC glycosynthases

the first-generation catalyst displayed low promiscuity toward some lipid acceptors including the phytosphingosine derivative. A new catalyst, D314Y/E351S mutant of EGC II discovered by directed evolution, was shown to have the desired high promiscuity.[215]

An interesting application of glycosynthases which has attracted much attention in recent years is the endoglycosidase-catalyzed transglycosylation for the synthesis of homogeneous glycopeptides and glycoproteins.[216] The *endo*-β-*N*-

acetylglucosaminidases (ENGase), which catalyze the hydrolysis of the glycosidic bond in the chitobiose (GlcNAcb1-4GlcNAc) skeleton, also possess transglycosylation activity that can transfer en bloc various types of *N*-glycans onto different acceptors. Two ENGases, Endo-A from *Arthrobactor protophormiae* and Endo-M from *Mucor hiemalis*, have been widely used to attach a large oligosaccharide moiety to a preformed GlcNAc peptide in a single step and in a regio- and stereoselective manner.[152,217] The relatively low tranglycosylation activity of ENGases prompted the development of other strategies including the use of synthetic sugar oxazoline as a highly active donor substrate.[152b,217c,218] To tackle the issue of product hydrolysis, especially when large full-size *N*-glycans were used, various mutants of Endo-A and Endo-M have been discovered where the hydrolysis activity was completely knocked out and the transglycosylation activity was enhanced.[217a,219] For example, a recent study by Yamamoto, Wang, and co-workers described an increased activity of Endo-M mutant N175Q for the synthesis of sialo-complex-type glycopeptides and glycoproteins.[220] A report by Wang and co-workers demonstrated an interesting one-pot enzymatic method, which is able to convert the high-mannose-type ribonuclease B into the complex-type ribonuclease C (Scheme 31).[221] In this approach, the sialylated *N*-glycan oxazoline was obtained by a semisynthetic method, wherein the free *N*-glycan released from isolated sialoglycopeptide (SGP) was treated with 2-chloro-1,3-dimethylimidazolinium chloride (DMC) to afford the oxazoline product quantitatively.[221] By taking advantage of the distinct substrate specificity of Endo-A and Endo-M, the high-mannose *N*-glycan of RNase B was first hydrolyzed by Endo-A and then glycosylated with EndoM-N175A by using **165** as the donor substrate to give RNase C in 70% yield in a one-pot manner.



Scheme 31. One-pot conversion of RNase B into sialylated RNase C by Endo-A-catalyzed hydrolysis and EndoM-N175A-catalyzed transglycosylation.

6. Application of Glycan Microarrays for Studying Human Diseases

Given the diverse and ubiquitous presence of glycans on cell surfaces, molecular recognition between carbohydrates and glycan-binding proteins (GBPs) is involved in a wide range of biological processes. The study of carbohydrate–protein interaction is, however, hampered by the low affinity of carbohydrates for glycan-binding proteins (GBPs), of which the monomeric K_D values are typically in the micromolar to millimolar range.[222] Glycan microarrays can be developed through immobilization of glycans onto a surface, and provide a direct mimic of the multivalent display of carbohydrate epitopes on the cell surface.[223] Only a small amount of glycans (a few nanoliters of solutions in concentrations of 1–100 μM) is needed, thus offering a relatively easier access to the preparation of glycan libraries.

However, preparing a broad glycan library remains a key challenge for the development of a glycan microarray. In general, there are two ways to obtain glycans: either isolation from natural sources or through synthesis. The isolation of glycans from natural sources is a good way to exploit the whole glycome efficiently, although the availability of a specific glycan is often limited and precise structural characterization of each component is still problematic. Turnbull and co-workers recently reported an efficient method to generate heparan sulfate by partial digestion of tissue-derived heparan sulfate polysaccharide chains followed by chromatography fractionation.[224] This approach allowed structurally diverse natural saccharide libraries to be produced from heparan sulfate variants in a short period of time. Synthetic methods provide another means to acquire structurally well-defined glycans. Recent advances in carbohydrate synthesis such as the aforementioned "programmable one-pot glycosylation" and the introduction of solid-phase oligosaccharide synthesis (SPOS),[225] especially automated solid-phase synthesis,[124a] have greatly reduced the labor needed for preparing glycans. However, it needs to be pointed out that the synthesis of oligosaccharides with very complex structures in a fully automated mode remains a major challenge.[3i]

Combining chemical and enzymatic syntheses can efficiently elaborate glycans with great diversity. This strategy has been used to elongate glycan structures through different glycosyltransferases working on the slide surface.[226] However, it is difficult to characterize and quantify elongated glycans on the array surface directly. Therefore, the glycan microarray prepared by this approach usually lacks quality control. To tackle this problem, Mrksich and co-workers developed a method in which a self-assembled monolayer combined with matrix-assiated laser desorption/ionization

time-of-flight mass spectrometry (SAMDI-TOF MS)[227] was used to observe and optimize glycan structures on the gold surface. This strategy was further used by Flitsch and co-workers to monitor glycosyltransferases involved in protein *O*-glycosylation.[228] Wong and co-workers developed a desorption/ionization on silicon mass spectrometry (DIOS-MS) method—an ionization method that uses a porous silicon surface to generate gas-phase ions of small molecules (< 3000 Da) without a matrix[229]—to characterize the oligosaccharides that are covalently bound to porous silicon with a built-in photocleavable linker.[47b] Nevertheless, it is quite tedious to incorporate the photocleavable linker into individual glycans. To tackle this problem, a technique based on nanostructure-initiator mass spectrometry (NIMS) was developed to characterize noncovalent glycan arrays by using fluorous-tagged substrates that are physically adhered to the fluorohydrocarbon-coated porous silicon surface.[230] However, the preparation of porous silicon plates requires corrosive acids, which may not generate uniform results. Recently, Wong, Wu, and co-workers developed a stable polyfluorinated aluminum-coated glass (ACG) slide through phosphonate reactions. This slide was then arrayed with polyfluorinate-tailed glycans to form a noncovalent array (Figure 14).[231] Since the ACG slides feature a conductive surface,[232] the noncovalent array can be characterized through mass spectrometry by ionization/desorption at low laser energy without addition of matrix. This type of array is very suitable for studies on enzyme activity and specificity. A representative cellotetraose array was developed to study the activity and specificity of different cellulases and to differentiate the activities of exo- and endoglucanases.[231] Similarly, a covalent glycan array on ACG was also developed (Figure 14) by spotting the glycan phosphonate directly on the ACG surface.

Another excellent combinatorial chemoenzymatic approach was introduced by Chen and co-workers.[233] An array of 72 sialosides were systematically synthesized by using a combination of 18 ManNAc/mannose substrates and 4 different biotin-attached acceptors in a one-pot three-enzyme synthesis. The products were transferred to a NeutrAvidin microtiter plate and used directly to study sialic acid binding lectins without purification, thus offering a high-throughput screening platform.

Glycan microarrays nowadays have a wide range of applications, including the study of enzyme activities,[234] investigations on the specificities of lectins[235] and antibodies,[236] monitoring antibody titers after immunization for vaccine development,[237] drug discovery,[238] serum antibody profiling,[239] and monitoring infectious diseases. The ultimate goal is to develop diagnostic or therapeutic approaches to target human diseases.

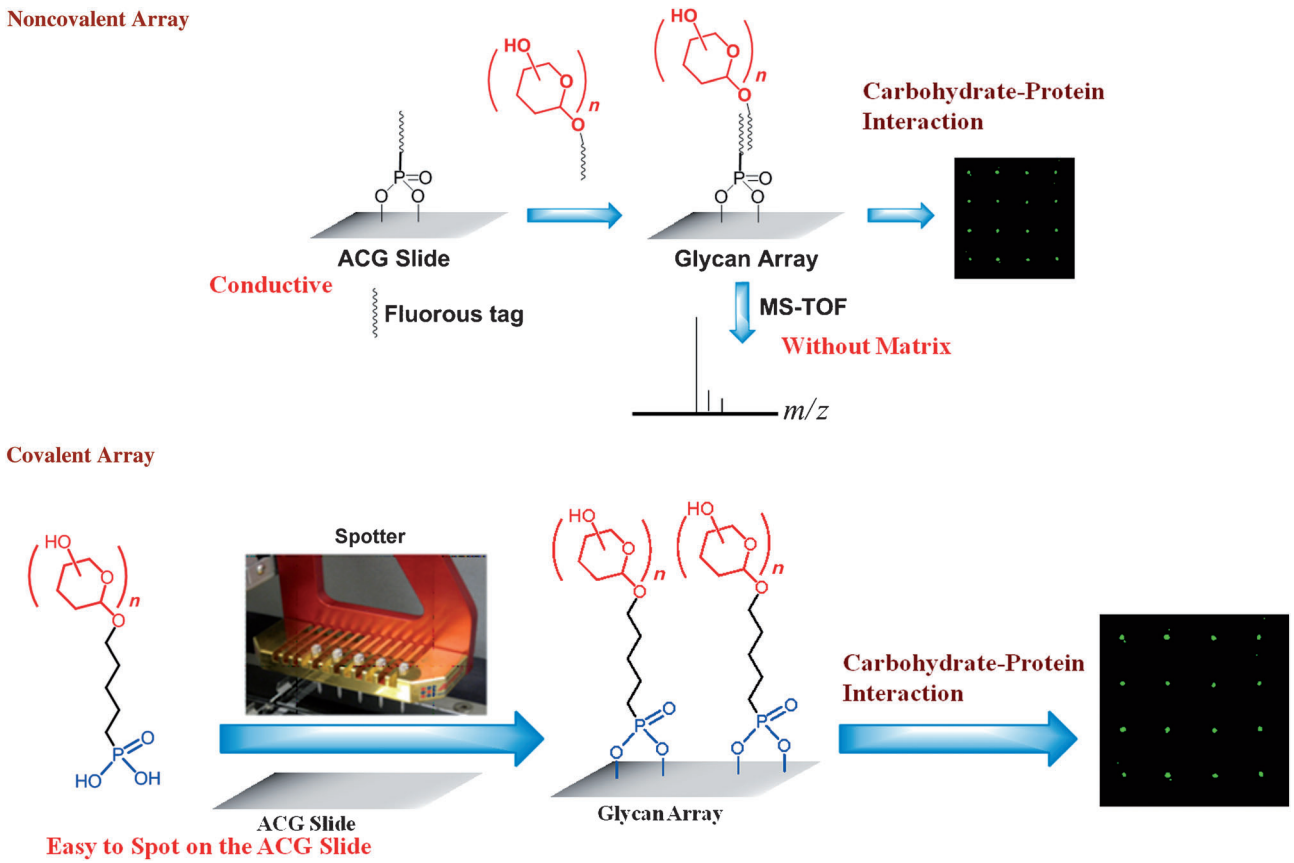


Figure 14. Glycan array on an aluminum-coated glass (ACG) slide.

6.1. Glycan Microrrays To Investigate Interactions between Sialosides and Influenza Hemagglutinin

Infection by the influenza virus is initiated by attachment of the virus to cell-surface sialoside receptors through one of the glycoproteins of the influenza surface, hemagglutinin (HA). Depending on its receptor specificity, influenza HA can be generally categorized into two kinds: human-adapted virus HA with preference toward Neu5Acα(2→6)Gal moieties and avian-adapted virus HA with preference toward Neu5Acα(2→3)Gal moieties. Such a subtle difference in the receptor specificity contributes to a species barrier which protects humans from infection by the avian influenza virus. However, mutations on HA might result in cross-species infection from the avian-adapted virus to the human-adapted virus. Therefore, it is of great interest to study HA receptor specificities in the hope of developing surveillance methods for the early detection of influenza viruses, especially in pandemic strains.[240]

Glycan microarrays have emerged as a powerful tool to study HA specificities.[223b] In 2006, Wilson, Paulson, and co-workers introduced a series of influenza H1, H3, and H5 glycan microarrays by using a library consisting of more than 260 glycans, which were developed by the Consortium of Functional Glycomics (CFG) at the Scripps Research Institute.[241] In these studies, prominent differences in the receptor-binding specificities were revealed in different HA subtypes for the structure features of oligosaccharides, including modifications such as sialylation, sulfation, and fucosylation of the vicinal glycan structures beyond the anomeric linkage of the terminal sialic acid. These findings demonstrated the sensitivity and high-throughput capability compared to haemadsorption and haemagglutination inhibition assays.[240] Glycan microarrays have become an essential tool for studying influenza virus receptor specificity of seasonal and pandemic strains, which includes the recent outbreak of a swine-origin H1N1 influenza A virus strain in 2009.[242]

Assessing the affinity of carbohydrate-binding properties is usually difficult due to the limited sources of glycans. There is a need to develop an accurate and quantitative method for glycan microarrays to investigate receptor specificities. Wong and co-workers recently reported a quantitative glycan microarray method,[243] which described the determination of the surface dissociation constants ($K_{D,surf}$), which can also be extended to determine the Gibbs free energy in protein–glycan binding. This quantitative glycan microarray was used to determine the surface dissociation constants ($K_{D,surf}$) of influenza H5 hemagglutinin and its ligands (Figure 15).[244] By comparing the change in ΔG ($\Delta\Delta G$) upon binding of HA to Neu5Acα(2→3)Gal disaccharide and four different Neu5Acα(2→3)Gal trisaccharides (β4- and β-3 GlcNAc, β4-Glc, and β3-GalNAc), the binding energy on different sialosides can be calculated and compared to determine the contribution of the constituents of the glycan structures to the HA–glycan binding energy and thus identify the best ligand (Figure 15a).

To quickly respond to a potential outbreak, it is of great importance to develop an effective diagnostic method capable of simultaneously typing and subtyping influenza viruses. Recently, Wong and co-workers initiated a study to subtype influenza viruses through the use of a glycan microarray containing a library of 27 synthetic sialosides.[242b] The glycan binding results of recombinant HAs from seasonal or pandemic viruses suggested a set of glycans including glycan numbers 24, 29, and 30 that are useful for subtyping H1 and H3 (Figure 16). All H1 HAs from pandemic and seasonal strains showed length-dependent binding specificity with a preference for longer α(2→6) glycans (Figure 16a–c). Similar to H1 HAs, the H3 HA from seasonal strain also showed strongest binding to the longest glycan (no. 30), which contains three LacNAc repeats (Figure 16d). However, for glycan 29, which contains two LacNAc repeats, only a very weak binding was found. A similar trend was observed in the profiling of the real virus isolates of corresponding strains.[242b] These results may form a basis for developing a glycan microarray diagnostic tool to differentiate influenza subtypes.

In addition to influenza virus, glycan microarrays can be used to detect various microorganisms such as *Bacillus anthra*cis, *Burkholderia pseudomallei*, and *Francisella tularensis*,[245] since they are known to carry a wide variety of glycan-binding proteins on the cell surface.

6.2. Glycan Microrray Applications in Cancer Diagnosis and Vaccine Development

Aberrant glycosylation is a hallmark of tumors; therefore, identification of uniquely expressed tumor-associated carbohydrate antigens (TACAs) has become an attractive approach for the development of new therapeutics and diagnostics for cancers (see Section 7.3). To date, diagnostics for the detection of cancer generally rely on genetic tests, serological methods, and other invasive detection tools such as endoscopy. On the other hand, prostate specific antigen (PSA) is widely used as a biomarker for the early diagnosis of prostate cancer. It will be interesting to see if TACAs can be used as an array for the diagnosis of cancer. To test this hypothesis, Wong and co-workers utilized a library of synthetic globo H and its fragments as an array to measure the level of anti-globo H antibody from the serum of breast cancer patients,[237a] and it was found that the binding specificity of sera from patients and normal donors were differentiated with statistical significance. Nevertheless, because the immune responses vary within individuals, some results displayed only subtle differences. The combined use of other TACAs with globo H and derivatives in a single glycan microarray may be considered for future development. High-throughput profiling of human serum by glycan microarray methods has also been developed, and a novel human cellulose-binding antibody has been identified.[246] The repertoire of anti-carbohydrate antibodies in patients transplanted with fetal pig islets was also characterized by using a glycan microarray.[239a]

Glycan microarrays are also a potential tool for the development of vaccines. Seeberger and co-workers have prepared a glycan microarray comprising synthetic *Plasmodium* glycosylphosphatidylinositol (GPI) glycans.[239b] This array was applied to compare the anti-GPI IgG levels in

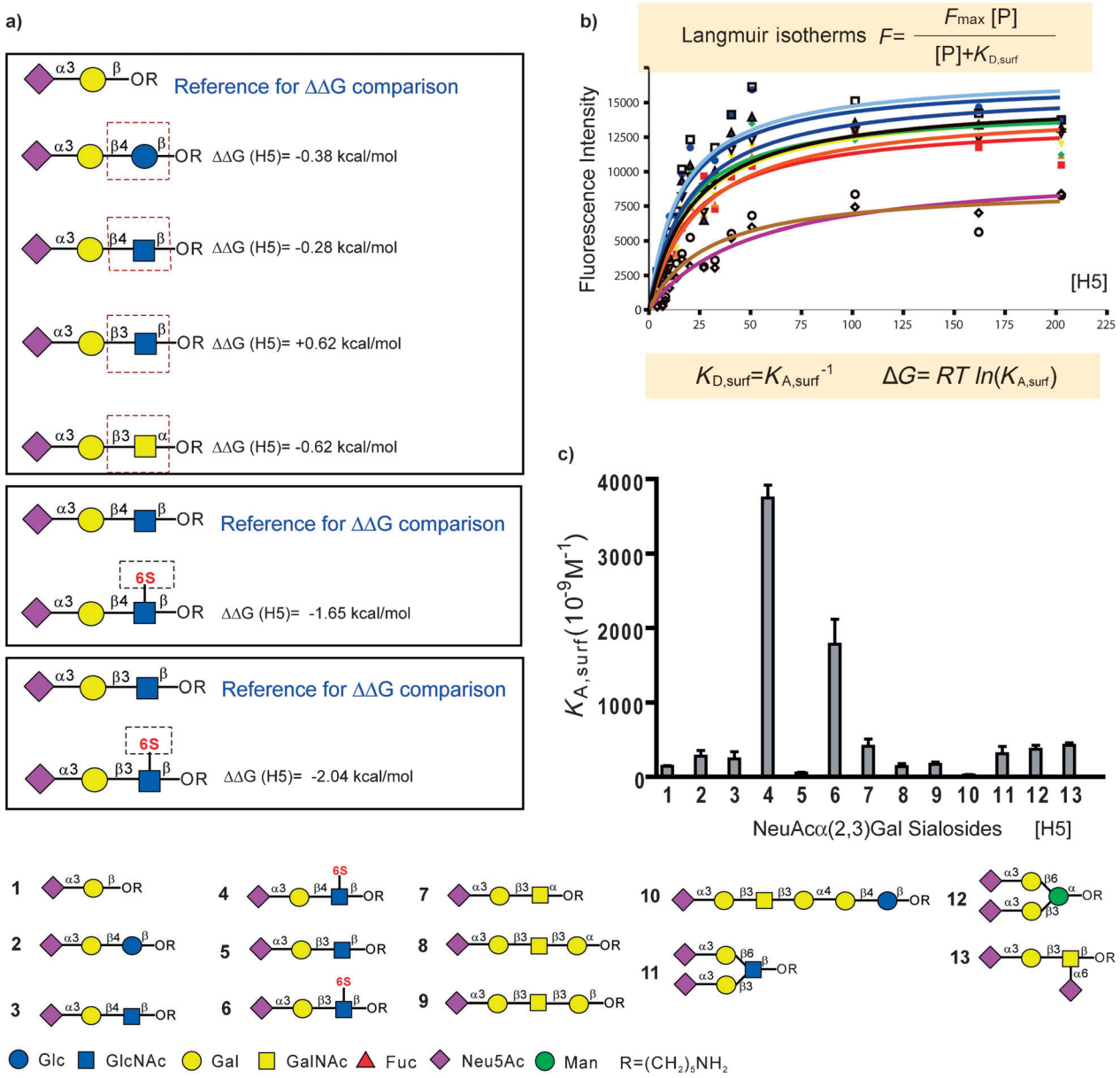


Figure 15. a) The binding energy contributions from sugars or modifications of H5HA–glycan interactions. b) Langmuir isotherms used to calculate $K_{D,surf}$. c) Association constants of H5 in response to α(2→3)-linked sialosides nos. 1–13.

donors from malaria-endemic areas with non-exposed individuals. The results showed that malaria exposure can have a great effect on the level of anti-GPI antibody. An array with 46 oligosaccharides has been used to identify the ligands for antibody MG96, which is a transmission-blocking agent used to block the development of parasites in mosquitos.[247] Several ligands were identified that could serve as components of putative antigenic epitopes.

Although glycan microarrays have been a key tool for glycobiologists, challenges still remain, such as the density of the immobilized glycans, which can greatly affect carbohydrate–protein recognition. Gildersleeve and co-workers evaluated the density-dependence on the binding of lectins and antibodies, and found that different antibodies recognized different densities of the same glycan antigen.[248] Recently, Wu and co-workers found that the antibody-binding profiles were affected by the density and structures of neighboring glycans in carbohydrate–antigen recognition.[249] Moreover, it was found that antibodies are capable of forming multivalent interactions with different glycans simultaneously, and the overall binding affinity increased in the presence of heterogeneous glycans.[249] The finding of heteroligand binding provides a new direction to the design of carbohydrate-based vaccines by using mixed antigens in a combinatorial fashion. The development of glycan arrays that mimic the natural system will gain more attention in the near future.

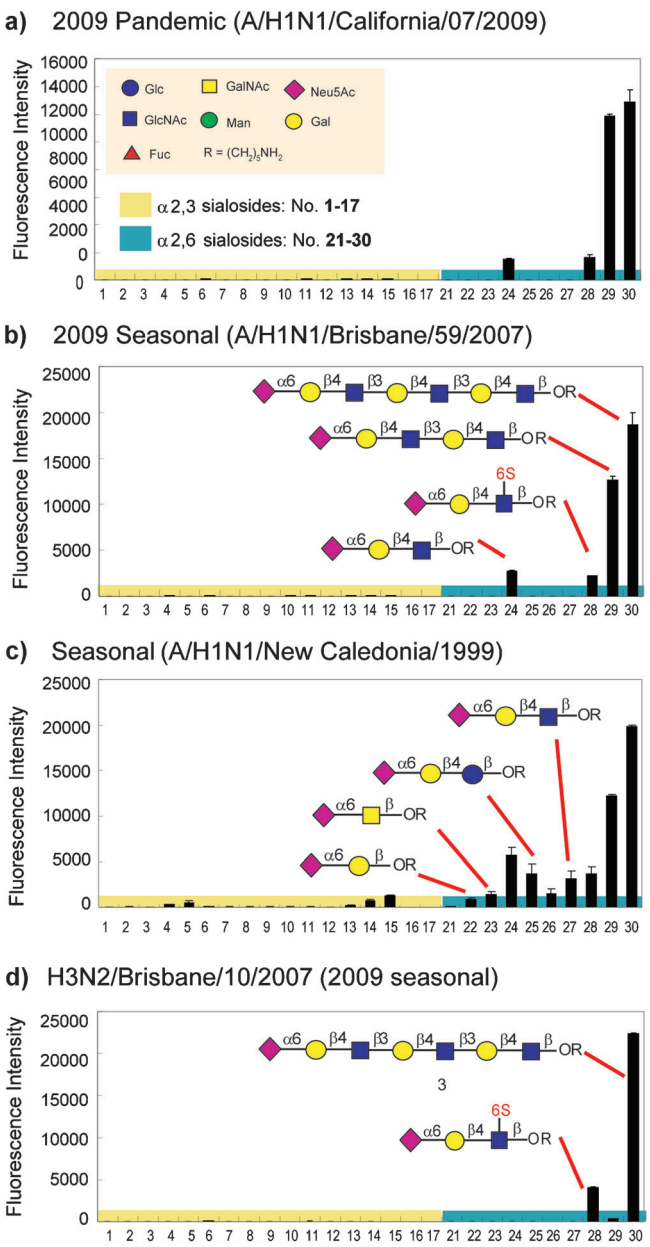


Figure 16. Differential binding patterns of HAs from H1N1 and H3N2.

6.3. Consortium for Functional Glycomics (CFG)

The CFG, one of the largest glycan microarray resources, was funded by the National Institutes of General Medical Sciences. The success of the CFG approach is a result of the combined efforts and generous input from many investigators in the field (*http://www.functionalglycomics.org*). Scientists have access to the CFG database for viewing CFG raw and summary data. The current CFG array (version 5.0) has 611 highly diverse glycans attached to NHS-derivatized glass microscope slides through amino-functionalized linkers. Some recent examples of applications of the array include the screening of mAb KEN-5,[250] Siglec-8 ligand,[251] glycosyltransferase and sulfotransferase gene expression,[252] and galectin-1 that recognizes high mannose on HIV-1.[253] Comparison of the receptor-binding capabilities of natural and mutated human and avian influenza viruses was also conducted by using the CFG array.[254] The CFG arrays have become important tools in the detection of antigens and the development of vaccines, and have provided a wealth of information on ligand specificities of the glycan-binding proteins.

7. Application of Synthetic Carbohydrates for Vaccine Development

The unique carbohydrate structures on the surface of invasive pathogens and the aberrant glycosylation on malignant cells make such carbohydrate moieties attractive immunotherapy targets. The rationale dates back to 1923 when Avery and Heidelberger reported that the pneumococcal antigens targeted by the immune system are polysaccharides.[255] On the basis of this study, Francis and Tillet observed in 1930 that intradermal injections of type-specific capsular polysaccharides (CPS) of pneumococci elicited polysaccharide-specific serum antibodies in healthy adults.[256] Additionally, Heidelberger and co-workers noted that pneumococcal capsular polysaccharides could be used as immunogens which offered type-specific long-lasting immunity against pneumococcal infections.[257] These developments form the basis of the potential use of carbohydrates, such as bacterial capsular polysaccharides, as vaccines. Their therapeutic potential are further highlighted by a number of commercially available carbohydrate-based vaccines against bacterial infections (Table 5), and by many promising vaccine candidates against human infection diseases and various types of cancer.[258]

Unfortunately, carbohydrates themselves are poorly immunogenic mainly because carbohydrate-mediated antibody production is T-cell-independent (TI), which results in the production of predominately low affinity, non-class-switch IgM antibodies and lack of immunology memory.[259] In addition, infants and children under the age of two respond poorly to TI antigens; immunity toward TI antigens normally requires two to three years after birth to develop. By contrast, proteins are immunogenic from early childhood; therefore, the conjugation of oligosaccharides to an immunogenic carrier protein could possibly convert the non-immunogenic carbohydrate antigens into immunogens. In this way, carbohydrate–protein conjugates can be digested by antigen-presenting cells and present the glycopeptide fragments to the MHC-II complex. This antigen complex stimulates a T-helper cell response, which facilitates B-cell maturation into antibody-secreting plasma cells and immunological memory cells.[260] Indeed, back in 1930, Avery and Goebel had demonstrated that coupling pneumococcal capsular polysaccharides to the carrier protein could induce a type-specific antipneumococcus response, which none of its constituents alone is capable of inciting.[261] Several commercially available antibacterial vaccines mentioned above are also based on conjugates.[258a]

Although natural polysaccharides have been used successfully as vaccines,[262] they show significant heterogeneity, which increases the difficulties in reproducibility. This issue

Table 5: Existing polysaccharide and conjugate vaccines on the market.[a]

Organism	Trade name	Vaccine type (component)	Manufacturer
Haemophilus influenzae Typ b (Hib)	HibTiter (1990)	**C** (Hib CPS with CRM197)	Wyeth
	PedvaxHIB (1990)	**C** (Hib CPS with OMPC)	Merck & Co.
	ActHib (1993)	**C** (Hib CPS with TT)	Sanofi Pasteur
	QuimiHib (2004)	**C** (synthetic Hib CPS with TT)	Vabiotech
	Hiberix (2009)	**C** (Hib CPS with TT)	GSK
	Pentacel (2008)	**C** (DT, TT, acellular pertussis adsorbed inactivated poliovirus, and Hib-TT conjugate vaccine)	Sanofi Pasteur
	Comvax (1996)	**C** (Hib-OMPC, conjugate and hepatitis B (recombinant) vaccine)	Merck & Co.
Streptococcus pneumonia (Pn)	Pneumovax (1983)	**P** (CPS of 23 Pn serotypes, 1, 2, 3, 4, 5,6B, 7F, 8, 9N 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, 33F)	Merck & Co.
	Prevnar (2000)	**C** (CPS of 7 Pn serotypes,4, 6B, 9V, 14, 18C, 19F 23F with CRM197)	Wyeth
	Prevnar 13 (2009)	**C** (CPS of 13 Pn serotypes, 1, 3, 4, 5, 6A, 6B, 7F, 9V 14, 18C, 19A, 19F, 23F with CRM197)	Pfizer
Neisseria meningitidis (Nm)	Menomune-A/C/Y/W-135 (2009)	**P** (group A, C, Y, W-135 CPS)	Sanofi Pasteur
	Menactra (2008)	**C** (group A, C, Y, W-135 CPS with DT)	Sanofi Pasteur
	Menveo (2010)	**C** (group A, C, Y, W-135 CPS with CRM197)	Novartis
Neisseria meningitides Group C (NmC)	Menjugate (2000)	**C** (group C CPS with CRM197)	Novartis (Chiron)
	Meningitec (1999)	**C** (group C CPS with CRM197)	Wyeth
	NeisVac C (1999)	**C** (de-*O*-acetylated group C CPS with TT)	Baxter
Salmonella typhi	Typhim Vi (1994)	**P** (Vi CPS)	Sanofi Pasteur

[a] **P**: polysaccharide vaccine, **C**: conjugate vaccine; CPS = capsular polysaccharide; OMPC = outer membrane protein complex (derived from *Neisseria meningitides*); DT = diphtheria toxin; CRM197 = diphtheria toxin mutant; and TT = tetanus toxid.

can be addressed by synthetic methods, which can generate specific carbohydrate antigens in a pure state and in relatively large amounts.[3i] In addition, since the immunogenic epitope often comprises only part of the saccharide structure (e.g. antibodies recognize epitopes that are no longer than a hexasaccharide), the availability of efficient synthetic methods opened the possibility to map the effective structure parameters (e.g. the oligosaccharide length) for the construction of vaccine candidates. The first synthetic oligosaccharide conjugate vaccine **166** (Quimi Hib), licensed in 2004, illustrates the potential use of organic synthesis for the preparation of carbohydrate-based vaccines (Figure 17).[263] This vaccine provided a comparable protection in the fight against the bacterium *Haemophilus influenzae* type b (Hib) as already licensed vaccines which incorporate the natural capsular polysaccharides.

Apart from the carbohydrate chemistry, a crucial step in the synthesis of glycoconjugate vaccines is to attach carbohydrate antigens to the immunogenic carriers. It is of great importance that the synthesized oligosaccharides be equiped with linkers bearing the proper functional groups for selective conjugation without affecting the hydroxy groups of the saccharide.[148,264] For example, the design of carbohydrate-based vaccines commonly utilize the terminal olefin contained in allyl or pentenyl glycosides as a linker for bioconjugation.[265] Conveniently, the oligosaccharides equipped with such functionality can be manufactured with the current automated oligosaccharide synthesis.[124a] Besides conjugation methods, other considerations that could influence the immune response to the carbohydrate moiety should also be taken into account in the design of glycoconjugate vaccines, including the types of terminal linkages between the saccharide and aglycon, the density and the length of saccharide chains, and the geometry and immunogenicity of the linker.[264a]

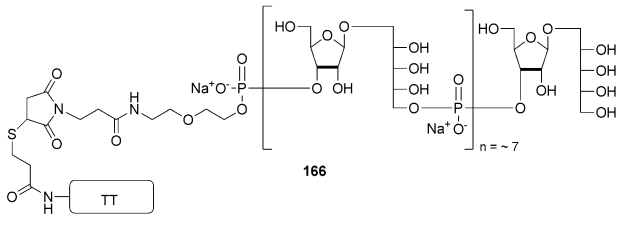
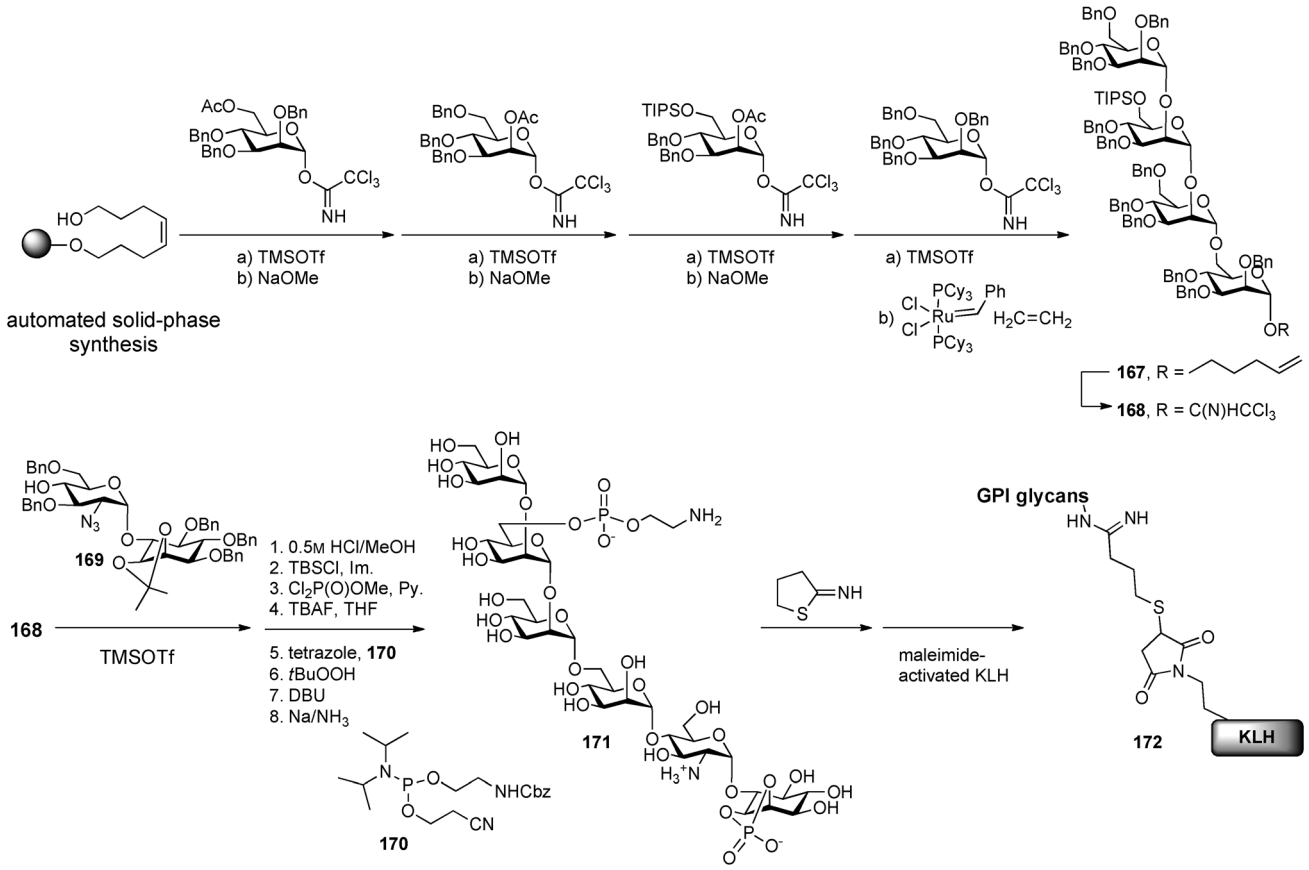


Figure 17. Synthetic vaccine for *Haemophilus influenzae* type b (Hib).

In the following part of the Review we attempt to summarize the biological application of the aforementioned synthetic methods in the development of carbohydrate-based vaccines. Rather than covering all aspects of this fast moving area, our focus is confined to carbohydrate-based antiparasitic, anti-HIV, and anticancer vaccines. For the discussion of carbohydrate-based antibacterial vaccines, the reader is referred to other specific reviews.[266]

7.1. Antiparasitic Vaccine Candidates

Similar to bacteria, many parasites synthesize unusual glycoconjugates on their surface that are often antigenic and



Scheme 32. Synthesis of *P. falciparum* GPI-based malaria conjugate vaccine through the combination of automated solid-phase and solution-phase oligosaccharide synthesis.

may be used as targets for developing antiparasitic vaccines.[267] *Plasmodium falciparum* is a protozoan parasite that is responsible for 95% of the malaria deaths worldwide.[268] Protein glycosylation in the *Plasmodium* is 90% highly conserved through attachment of a glycosylphosphatidylinositol (GPI) anchor, which is implicated as a key parasite toxin that induces macrophages to produce proinflammatory cytokines and nitric oxide, thus contributing to malaria pathogenesis.[269] Seeberger and co-workers explored the possibility of employing GPI as a target by synthesizing the vaccine candidate **172** containing a hexasaccharide of the *P.* falciparum GPI glycan in conjugation with the carrier protein (Scheme 32).[270] The tetramannosyl fragment **168** of GPI can be synthesized by using the automated solid-phase method,[271] followed by coupling with the core disaccharide **169** in solution. The resulting hexasaccharide was coverted into malaria toxin **171**, treated with 2-iminothiolane to introduce a sulfhydryl group at the ethanolamine, and conjugated to maleimide-activated keyhole limpet hemocyanin (KLH), a large immunogenic protein obtained from the blood of a shellfish. The conjugate **172** was then administered to mice. Significant protection was observed against *P. berghiei* induced pathology, such as malarial acidosis, pulmonary edema, and cerebral syndrome. Although mortality is reduced, the parasitaemia level was unaltered between the vaccination and control groups, thus indicating that the anti-GPI antibody response does not interfere with the parasite replication, but specifically blocks the virulent GPIs. This study is currently undergoing clinical evaluation by Ancora Pharmaceuticals (Medford, MA, USA).[272] It is also noted that the synthetic GPI structures[273] of *Plasmodium* offer not only the possibility of vaccine development but also basic research on malaria immunopathogenesis.[239b]

Another potential protozoan parasite target is the GPIs of *Toxoplasma gondii*, a causative agent of toxoplasmosis, which causes severe infection to the fetus by primordial infection during pregnancy.[274] A detailed structure analysis identified two GPIs structures that were widely distributed among *T. gondii*: (ethanolamine-PO_4)-Manα1-2Manα1-6(GalNAcβ1-4)Manα1-4GlcNα-inositol-PO_4-lipid and (ethanolamine-PO_4)-Manα1-2Manα1-6(Glcα1-4GalNAcβ1-4)Manα1-4GlcNα-inositol-PO_4-lipid, of which only the latter bearing the unique glucose-*N*-acetylgalactosamine side branch is immunogenic in humans.[275] It was shown that GPIs of *T. gondii* can activate macrophages to produce tumor necrosis factor α (TNFα) during toxoplasmal pathogenesis.[276] Therefore, it may be worth pursuing an antipathogenesis vaccine on the basis of these GPIs.

Leishmaniasis, a world-wide vector-borne disease affecting 88 countries and with an estimated 2 million cases annually, is transmitted to humans and other mammalian hosts by parasites in the blood-feeding sandfly.[277] This

disease is caused by a parasite belonging to the genus *Leishmania*, which contains multiple species and subspecies causing cutaneous, visceral, and mucosal leishmaniases. The epidemiology of leishmaniasis is extremely diverse, thereby complicating its treatment.[278] In all species of *Leishmania* that infect humans, the dense glycocalyx contains phosphosaccharide repeat units, of which lipophosphoglycan (LPG) represents one of the major surface molecules and is an important virulence factor.[279] The LPG structures of different *Leishmania* species share a conserved organization, which comprises a GPI anchor constituted by a lipid domain and core glycans, a repeating 6Galβ(1→4)Manα1-PO_4- disaccharide and a small oligosaccharide cap (Figure 18a).[280] The

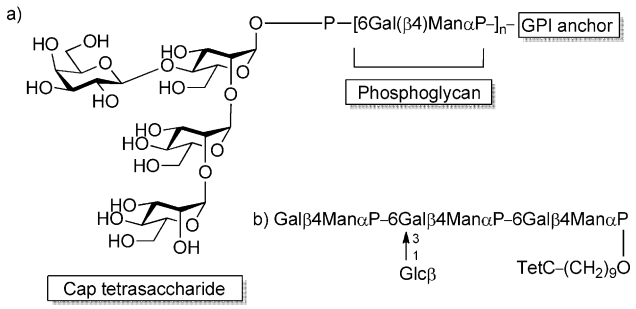


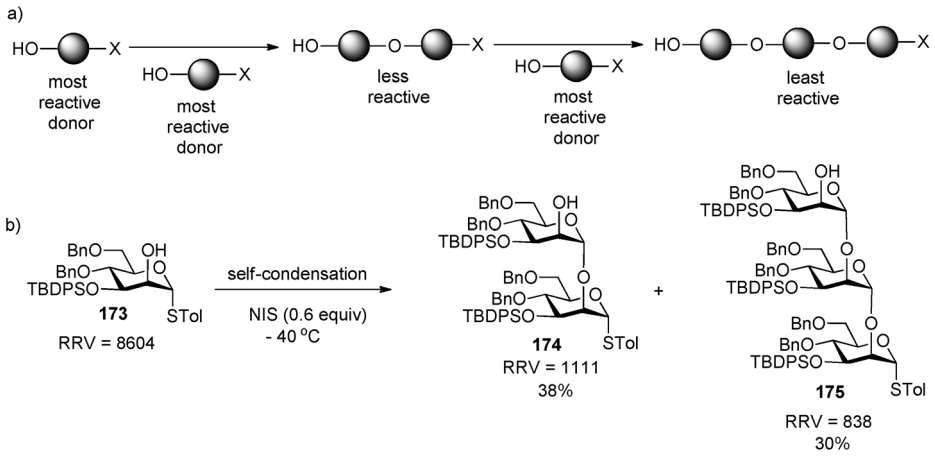
Figure 18. a) A General structure of *Leishmania donovani* lipophosphoglycan (LPG). b) Chemical structure of the glycovaccine-containing phosphoglycan repeating structure of *L. mexicana* in conjunction with recombinant tetanus toxin fragment C (TetC). P = phosphate.

structure of the backbone repeating units and cap motif can be substituted to various extents with carbohydrate side chains or can stay unsubstituted depending on the species.[280] The terminal cap structure is important for the invasion of the parasite into macrophages.[281] The synthesis of the branched cap tetrasaccharide moiety through automated solid-phase method has been described by Hewitt and Seeberger.[282] Although immunological studies on Leishmaniasis carbohydrate vaccines manufactured by the automated synthesis remain to be completed, several synthetic vaccine candidates based on LPG structures have been described.[283] For example, Seeberger and co-workers synthesized the cap tetrasaccharide of *L. donovani*, which was equipped with a thio-based linker to couple the oligosaccharide moiety to the maleimide-derived influenza coat protein hemagglutinin.[283b] The resuting HA–glycan conjugate was formulated into a virosome[284] to give the HA–glycan-loaded immunostimulating reconstituted influenza virosomes (IRIVs) construct. The virosomal formulations resulted in both IgM and IgG antiglycan antibodies in immunized mice models, which cross-reacted in vitro with the natural carbohydrate antigen expressed by *L. donovani*. This result indicated that IRIVs have great potential for the development of carbohydrate-based vaccines, since they can induce T-cell-dependent antibody responses against oligosaccharide antigens.

In another study, Rogers et al. utilized the chemically synthesized phosphoglycans of various structures of *L. major*, *L. mexicana*, and *L. donovani* in conjunction with the TetC peptide of tetanus toxoid to produce four glycovaccines, which were used to immunize mice to be challenged by sandflies infected with *L. mexicana* (Figure 18b).[283c] Although none of them were able to protect against an injected artificial infection, the glycan from *L. mexicana* was protective against the challenge by the bite of infected sand flies, thus indicating that these glycoconjugate vaccines may induce species- and glycan-specific protection. Interestingly, this is the first demonstration that a synthetic glycoconjugate vaccine could exhibit a direct antiparasite effect in leishmaniasis.

7.2. Anti-HIV Vaccine Candidates

The pandemic of acquired immune deficiency syndrome (AIDS) worldwide, mainly caused by the infection of highly virulent human immunodeficiency virus type 1 (HIV-1), kills an estimated 2 million people annually. Currently, more than 30 million people are living with the disease. However, there are significant challenges for developing effective protective vaccines against AIDS, because HIV-1 viruses evolve several defense mechanisms to escape the immune surveillance by the host immune system.[285] One such obstacle is that the dense "glycan shield" coated on the surface envelope glycoprotein gp120 occludes the antigenic polypeptide and leads to immune tolerance. Intriguingly, however, the highly conserved high-mannose-type *N*-glycan clusters on the gp120 surface have been shown to be an alternative target for the development of an HIV-1 vaccine.[258a,285,286] This concept is attributed to the successful isolation of the broadly neutralizing IgG 2G12 from HIV-infected individuals. This antibody binds exclusively to the surface carbohydrates of gp120 and is able to neutralize a range of HIV-1 strains.[287] Mutational, biochemical, and structural studies of antibody–antigen complexes revealed that the epitope of 2G12 is mannose-dependent, in particular with specificity for terminal Manα-(1→2)Man moieties on the D1 and D3 arms of *N*-glycans.[236b,287b,c,288] Meanwhile, 2G12 exhibits an unusual swapped V_H domain structure to generate a multivalent antigen-binding surface which is unique to the bivalent interactions of typical immunoglobulins.[236b,288] These studies on the specificity of 2G12 have promoted the development of new vaccines, wherein the immunogen design was mainly based on mimicking the 2G12 epitpope, the oligomannose cluster, with an ultimate goal to elicit "2G12-like" antibodies. The classical design of the immunogen is to create oligomannose clusters, which are generated from the regioselective coupling of chemically pure oligomannose with the scaffolds to provide multivalency, and some of these glycoconjugates are further linked to protein carriers.[286b] Biochemical studies suggested that $Man_9GlcNAc_2$ and Man_4 are two favorable ligands for 2G12.[236b,289] Several chemical methods have been reported for the synthesis of these high-mannose glycans.[131b,136,290] For example, Wong and co-workers introduced a chemoselective one-pot strategy involving the self-condensation of thiomannoside monosaccharide to efficiently synthesize the Manα-(1→2)Man oligomannose of Man_4 (Scheme 33).[291] In this one-pot method, the most reactive monomer undergoes self-condensation to give a less-reactive dimer. The dimer then



Scheme 33. Synthesis of di- and trimannose donors by one-pot self-condensation.

serves as an acceptor for another monomer molecule, which leads to formation of the trimer (Scheme 33a). The best result was obtained using **173** with a nonpolar TBS protecting group on C3 and 0.6 equivalents of promoter NIS at −40 °C to achieve an optimal glycosylation (Scheme 33b). Several high mannose structures such as Man_4, Man_5, Man_7, Man_8, and Man_9 were easily synthesized by using di-**174** or trisaccharide **175** as the glycosylation donor.[236b] In addition, Danishefsky and co-workers described a total synthesis of $Man_9GlcNAc_2$, to provide direct access to mimics of the 2G12 epitope.[144,292]

Recent examples of new immunogen design include the coupling of oligomannose ligands to bacteriophage Qβ,[293] bovine serum albumin (BSA),[294] cyclic peptides,[295] and dendrimers[236c,296] (Figure 19). Some of these immunogens indeed display high affinity toward 2G12. For example, multivalent Man_9 glycodendrons (9-mer, Figure 19b), which was synthesized by a copper(I)-catalyzed cycloaddition, exhibited extraordinary inhibition of both gp120-2G12 and gp120-DC-SIGN in a nanomolar range. In addition, the bacteriophage Qβ conjugates of synthetic Man_4 and Man_9 (Figure 19c) interacted with 2G12 with nanomolar affinities, which makes them among the best 2G12-epitope mimics described thus far.

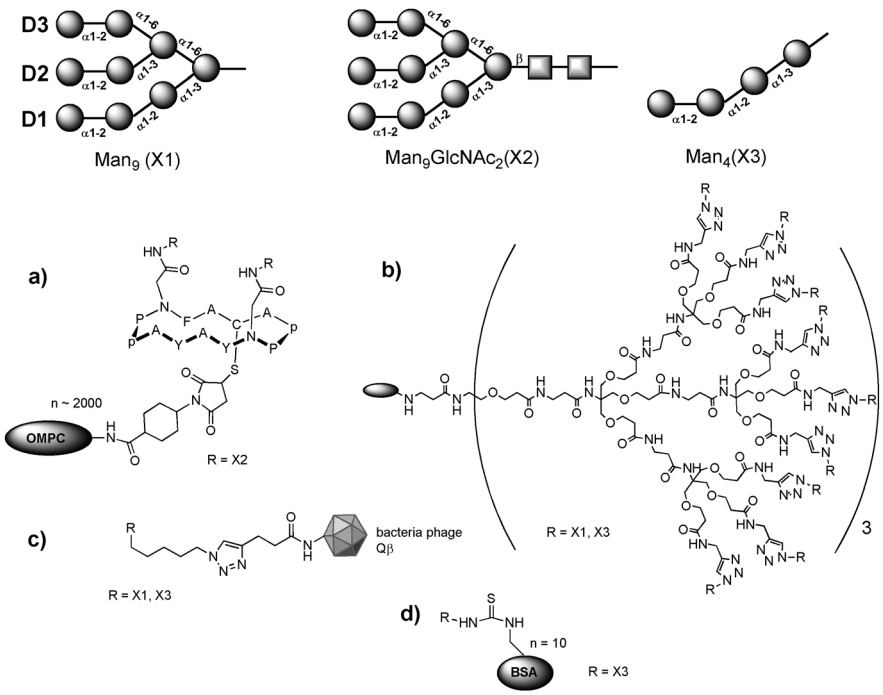


Figure 19. Synthetic oligomannose conjugates. a) Divalent glycopeptides–OMPC conjugate with $GlcNAc_2Man_9$, b) Man_4-, Man_9-glycodendron–BSA conjugate, c) Man_4-, Man_9-Qβ bacteriophage conjugate, d) Man_4–BSA conjugate. BSA = Bovine serum albumin, OMPV = outer-membrane protein complex.

In addition to utilizing oligomannose as the central immunogen, Wilson, Davis, and co-workers recently reported a novel design involving a nonself sugar mimic (Figure 20).[297] Unfortunately, so far, none of the antibodies elicited by these immunogens are able to neutralize HIV-1 virus.[258a] These results might reflect the improper display of current immunogen design, and the inability to induce domain exchange in the antibody produced, which is the unique architecture of 2G12. Since previous studies revealed that the polypeptide of gp120 may serve as a rigid scaffold to maintain the integrity of the spatial arrangement of 2G12-specific oligomannose clusters, the manner and flexibility of the connection to the scaffold is another important concern.[293] Recently, Scanlan and co-workers reported that the antisera immunized by α(1→3)-mannosyltransferase-deficient *S. cerevisiae* displayed remarkable similarity in its carbohydrate specificity to 2G12, and inhibited statistically significant, although extremely weak neutralization of the HIV-1 virus compared to control immune sera.[298] In addition, some of the glycoproteins expressed by *S. cerevisiae* strains that lack several carbohydrate-processing enzymes and produce specific $Man_8GlcNAc_2$ glycosylation displayed high affinity to 2G12 and inhibited gp120 interactions with 2G12 and DC-SIGN.[299] These findings suggested that the genetic modulation of microbial polysaccharides is an alternative route towards immunogens capable of eliciting antibody responses to the glycans of HIV-1.

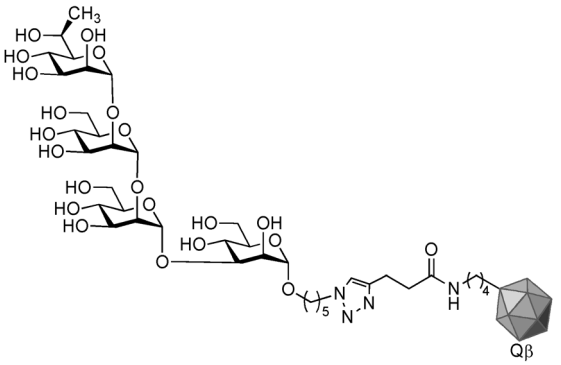


Figure 20. Nonself α(1→2)-linked Man_4 glycoconjugate.

as their structural similarity to normal antigens.[302a,b] Therefore, it will be difficult to break the tolerance which induces an insufficient immune response to eradicate the cancer. In addition, a serious problem associated with carbohydrate antigens is their poor immunogenicity and T-cell-independent feature. Another issue that one must consider is the overall low availability of such antigens.

The particularly poor immunogenicity of TACAs can be overcome by chemical conjugation to an appropriate carrier in the presence of a suitable adjuvant. Evidence has shown that a number of glycoconjugates of TACAs can elicit antibody responses against the corresponding carbohydrate moieties and were able to eradicate circulating cancer cells

7.3. Anticancer Vaccine Candidates

It has been known for several decades that tumor development is usually associated with changes in cell-surface carbohydrates.[300] The common aberrant glycosylation observed in human cancer includes the enhanced expression and altered carbohydrate structures of glycoproteins and glycolipids, for example, an increase in α(1→6) branching of *N*-glycans and elevated levels of sialylation and fucosylation.[1a,301] A broad range of saccharide structures have been characterized as potential tumor-associated carbohydrate antigens (TACAs),[302] which can be categorized into three groups: 1) mucin-related *O*-glycans, including Tn (GalNAcα-O-Ser/Thr), TF(Galβ1-3GalNAcα-O-Ser/Thr), and STn (Neu5Acα2-6GalNAcα-O-Ser/Thr); 2) glycosphingolipids, including gangliosides GM2, GD2, GD3, fucosyl GM1, and neutral globoside globo H; and 3) blood-group antigens, including SLe^x, Le^y-Le^x, SLe^a, and Le^y in *N*-linked, *O*-linked, or lipid-linked structures (Figure 21).

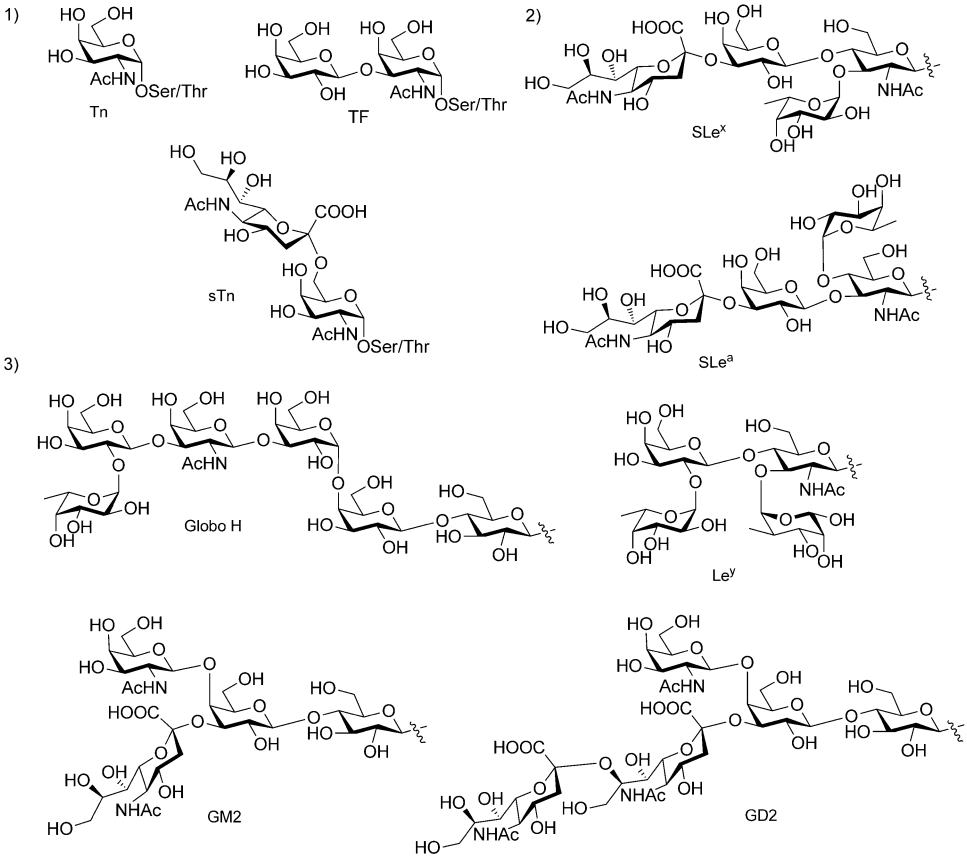


Figure 21. Structure of selected tumor-associated carbohydrate antigens (TACAs).

Cancer immunotherapy aims to boost a patient's own immune system to either treat cancers (therapeutic vaccines) or to delay the relapse of the cancer (prophylactic vaccines). Since TACAs are uniquely or excessively exposed on the surface of the cancer cell, it is of great interest to exploit TACAs therapeutically so as to elicit antibodies against TACAs and specifically eliminate malignant cells.[303] However, several studies have shown the existence of TACAs in normal tissues during specific stages of development, as well and micrometastasis in preclinical and clinical studies.[304] Moreover, the development of effective methods for the synthesis of TACAs is of particular importance for the development vaccines, as it would provide pure and reliable products.[305]

Currently, there are two strategies used for developing carbohydrate-based cancer vaccines: a) semisynthetic glycoconjugates and b) fully synthetic glycoconjugates (Figure 22). According to the number and type of carbohydrate epitopes linked to a carrier protein, strategy (a) can be further classified into 1) monovalent with a single type of TACA; 2) monovalent cluster containing a single type of TACAs;

3) unimolecular polyvalent constructs containing several types of TACAs.[306] Fully synthetic conjugates consist of 4) two-component, 5) three-component, and 6) four-component epitopes. In this part of the Review, we will summarize the most successful carbohydrate-based anticancer vaccines with selected TACA antigens. Recent reviews of this subject by Boons,[307] Guo,[308] Danishesky,[306] Ragupathi,[309] and Wu[183c] are recommended for further information.

7.3.1. *Glycosphingolipid-Based Vaccine Candidates*

Based on the successful demonstration of improved immunogenicity of conjugating bacterial polysaccharides to the immunologically active protein in the 1990s, researchers focused on the design of carbohydrate-based cancer vaccines by linking TACAs to a suitable carrier protein. Key studies by Livingstone and co-workers have systematically established the immunological effect of this conjugation.[310] Of several polypepetide carriers and adjuvants investigated, the covalent attachment of antigens to KLH through co-administration with immunoadjuvant QS-21, a saponin fraction derived from the bark of the South American tree *Quillaja saponaria molina*,[311] served as the most potent carrier and adjuvant pair to enhance immune response. In another comparative study in patients, GM2-KLH plus QS-21 also proved to be the most potent.[312] This combination has induced higher titers and more consistent antibodies in patients than the use of pure antigen with adjuvants.[313] Based on these striking findings, intensive efforts have been directed toward the preparation of various ganglioside-KLH conjugate immunogens, including

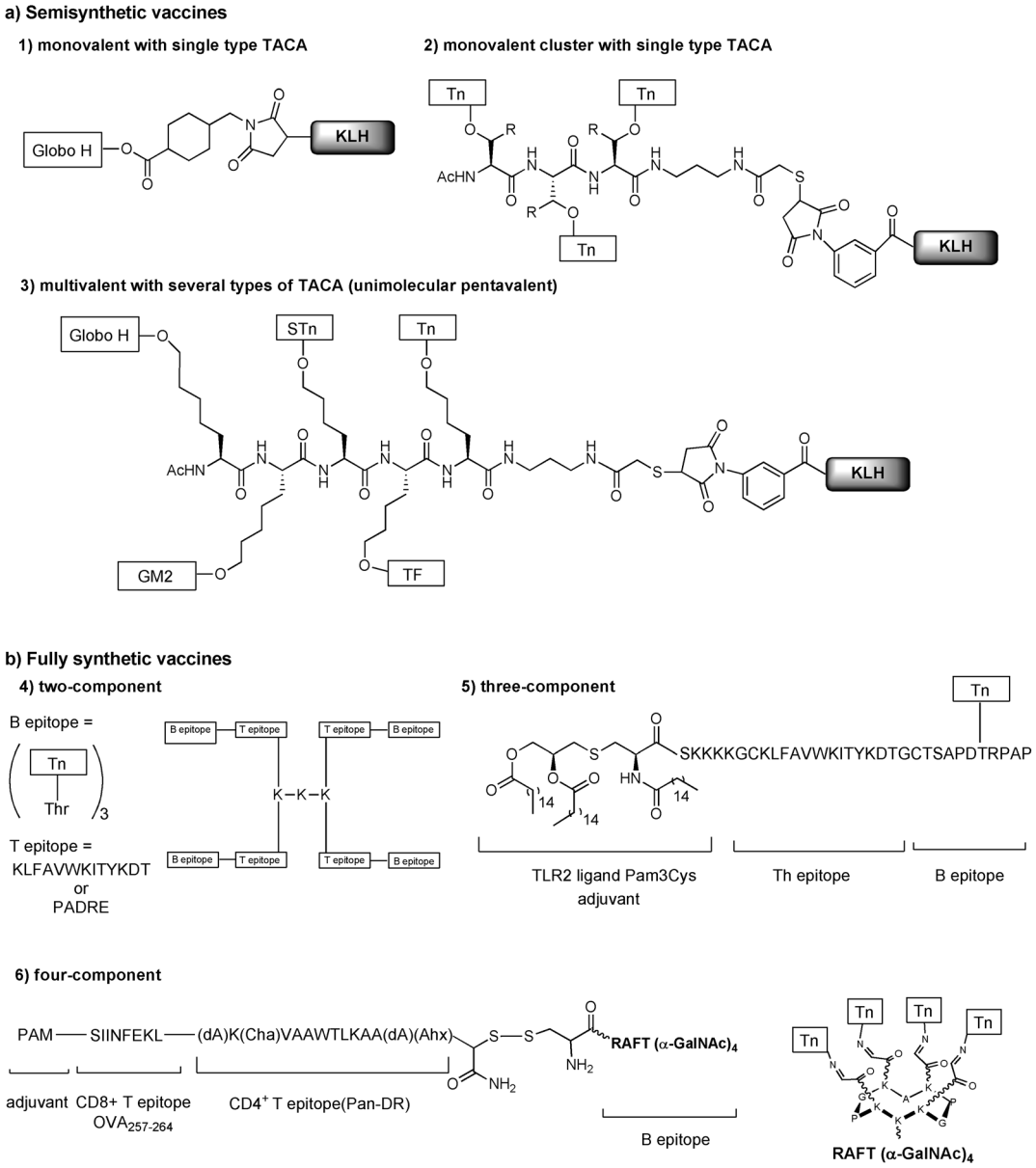


Figure 22. Various types of carbohydrate-based anticancer vaccine candidates. a) Semisynthetic vaccines with single or multiple TACAs coupled with an immunogenic carrier protein, b) fully synthetic vaccines with two or more covalently linked epitopes including B epitope, CD4+ epitope, CD8+ epitope, or built-in adjuvant.

Le^y-KLH, fucoyl GM1-KLH, GD2-KLH, GM2-KLH, GD3-KLH, and globo H-KLH.[309,314]

7.3.1.1. *Globo H*

Globo H is a tumor-associated antigen that is selectively expressed on a number of tumours, including breast, colon, ovarian, pancreas, and prostate.[39] While overexpressed in tumor cells, globo H is only weakly expressed in healthy tissue in the apical epithelial cells at lumen borders, a site that appears to be inaccessible to the immune system. This differential expression offered a promising possibility for exploring the use of globo H as a cancer vaccine target.[302b] The lack of a sufficient natural source, has resulted in globo H being the target of many total syntheses by many different methods. Danishesky and co-workers first reported the synthesis of such a complex structure by using a glycal assembly method.[315] Up to now, a number of efficient methods have been developed, including sequential,[316] automated solid-phase (Scheme 25),[128] one-pot (Figure 4 and Scheme 16),[40,44,317] enzymatic,[318] and chemoenzymatic[319] methods, thus providing a relatively easy access to globo H antigen. The first (globo H)–KLH glycoconjugate was prepared by ozonolysis of fully deprotected allyl glycoside followed by reductive amination with KLH.[320] Subsequent improvement based on the maleimide–thiol conjugation protocol has also been reported (Figure 22 a1).[321] Preclinical studies conducted with (globo-H)–KLH plus QS-21 in mice induced high IgM and IgG titers against globo H by ELISA.[322] Tests on patients, who received subsequent vaccination for relapsed prostate cancer after primary therapies, demonstrated that the vaccine is safe, with no significant toxicity detected.[323] The clinical effect was also demonstrated by the decline in the prostate specific antigen (PSA) levels for one third of the patients in pretreatment versus posttreatment. Studies also showed that the antibody response in breast cancer patients is similar to that described above in the prostate cancer patients.[321]

Recently, it was demonstrated that the diphtheria toxin (DT) CRM197, which has been widely used for human vaccination against diphtheria for decades and approved by the FDA for various glycoconjugate vaccines, is a promising carrier protein for the construction of an anticancer vaccine based on globo H.[324] A new vaccine candidate composed of globo H hexasaccharide in conjugation with DT CRM197 plus a glycolipid adjuvant showed an effective immune response, inducing more IgG antibodies against globo H than the other carrier proteins or glycolipid adjuvants used.[324] Intriguingly, the induced antibodies neutralized not only globo H but also Gb5 and SSEA-4, both are specifically expressed in breast cancer cells and cancer stem cells (Figure 23).

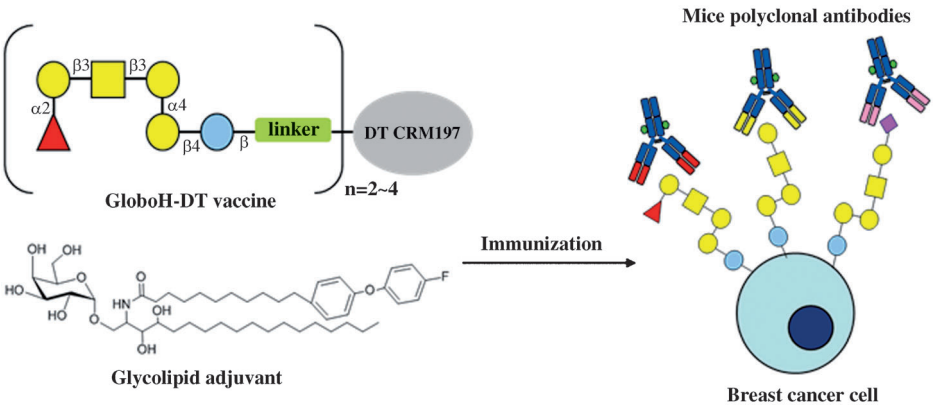


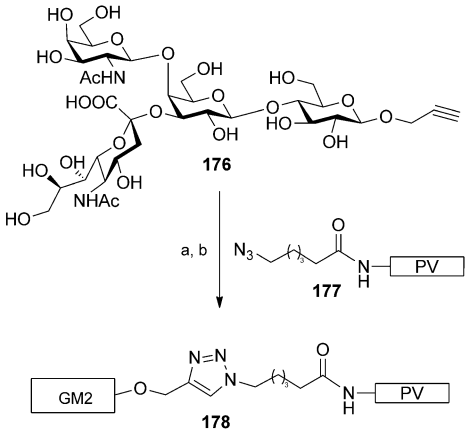
Figure 23. A new cancer vaccine composed of globo-H conjugated to diphtheria toxin CRM197 plus a glycolipid adjuvant.

Although there are several successful examples, some limitations of semisynthetic conjugate vaccines remain. First, the carbohydrate density achieved on protein glycoconjugates is highly variable. Thus, the resulting batch to batch variations may influence the clinical evaluation and vaccine efficacy. Moreover, the production of irrelevant antibodies against the carrier protein or linker may lead to carrier-induced epitopic suppression.[325] Therefore, the completely chemical synthesis of immunogens with its ability to generate accurate molecular definition of glycoconjugates in terms of their structure and composition has become an alternative strategy for the development of cancer vaccines.

7.3.1.2. *GM2*

It is known that GM2 ganglioside is widely expressed in many types of cancer including breast, ovary, prostate, small cell lung cancer, and melanoma.[302b] In a series of studies with the GM2–KLH in patients with malignant melanoma, Livingston and co-workers showed that vaccination with GM2 neoglycoprotein consistently induced high titers of GM2-specific IgM and durable IgG antibodies.[312] In addition, these antibodies were able to induce complement-mediated lysis of GM2-positive tumor cells.[326] These trials have demonstrated that enhanced GM2 antibodies have a clinical advantage. Loman and co-workers recently reported an alternative glycoconjugate of synthetic, structurally defined GM2 neoglycopeptides **178** (Scheme 34).[327] Interestingly, the conjugatable form of GM2 oligosaccharide **176** can be produced in gram-scale quantities by metabolically engineered *E. coli*. The resulting propargylated GM2 **176** was ligated with mono-azido-PV peptide **177** through copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition to give the desired GM2 glycopeptide **178**. The mice immunized with such short glycopeptides **178** were able to induce IgG and IgM antibodies against GM2 gangliosides. The sera from these mice could recognize human cancer cells expressing GM2. This glycopeptide represents a promising cancer vaccine strategy since the GM2 oligosaccharide antigen can be efficiently prepared and directly conjugated with the peptide carrier.

The metabolically engineered *E. coli* strains have been used to successfully synthesize (and in some cases with



Scheme 34. Synthesis of glycopeptide **178** by using propargylated GM2 produced by metabolically engineered *E. coli*. Reaction conditions: a) CuSO4, sodium ascorbate, H_2O/THF (1:1); b) RP-HPLC.

multigram production) conjugatable saccharide moieties of GM2 and GM3,[182] the carbohydrate moieties of several tumor-associated sialosyl oligosaccharides, such as GD3[183] and SLe^x,[181c] and globosides, such as globotetraose,[328] globopentaose,[329] and globo H.[330]

7.3.2. *O*-Glycan-Based Vaccine Candidates

The human mucine MUC1 is a membrane-bound glycoprotein. Its large extracellular domain contains various numbers of tandem repeats of 20 amino acids HGVTSAPDTRPAPGSTAPPA with 5 potential sites of *O*-glycosylation.[331] In a large variety of epithelial cancers such as breast, ovarian, pancreatic, and prostate, MUC1 is overexpressed and their *O*-glycans are incompletely processed, thereby resulting in aberrant glycosylation patterns, which are restricted to short carbohydrate chains such as Tn, TF, and the corresponding sialylated derivatives sTn and sTF (Figure 21).[332] These short carbohydrate side chains not only constitute cancer-associated antigens themselves but also expose previously masked peptide motifs, thus providing new antigenic targets based on MUC1 glycopeptides.

7.3.2.1. Tumor-Assoicated Mucin-Type O-Glycans

To closely resemble Tn cluster (c) expression on tumor mucins, glycoconjugates of trimeric Tn with KLH (Figure 22a2) and PAM (palmitic acid) have been synthesized[333] and tested[334] with QS-21 in a phase I trial in patients with relapsed prostate cancer. The use of lipid moiety PAM offered a chemically defined structure and could augment immunogenicity of both the peptide and carbohydrates. However, the KLH conjugate induced specific humoral responses (IgM and IgG), while fully synthetic Tn(c)–PAM induced IgM but failed to elicit IgG antibodies. The tri-Tn moiety was also used to construct a fully synthetic multiple antigenic glycopeptide (MAG) containing a four-arm non-immunogenic polylysine core with each arm extended by a mouse MHC class II restricted polio virus (PV) peptide (T epitope) carrying a tri-Tn glycotope (B epitope; Figure 22b4).[335] Immunization of this vaccine candidate mixed with mild adjuvant alum in mice induced Tn-specific IgM and IgG class antibodies with titers that were persistent for more than six months. To better understand the clinical effect of MAG compared to KLH, the immunogenicity of Tn(c)–KLH versus Tn(c)–MAG using different adjuvants was studied.[336] In contrast to Tn(c)–MAG, strong antibody response against KLH was induced when Tn(c)–KLH was administered with alum. Additionally, anti-Tn titers induced by the MAG conjugate are significantly higher than the KLH construct carrying the same tri-Tn glycotope. No immune memory response was evident after a boosting injection of KLH or MAG conjugates in the mice that initially received Tn(c)–KLH in QS-21. On the other hand, a memory response was elicited by previous Tn(c)–MAG immunization upon boosting injection. A similar MAG construct, in which the PV peptide was replaced by human MHC II restricted PADRE T_h epitope, was tested in nonhuman primates and induced anti-Tn IgG antibodies, which could mediate antibody-dependent cell-mediated cytotoxicity (ADCC) against tumor cells in the presence of human NK cells.[336]

Recently, Renaudet and co-workers took advantage of the synthetic versatility of regioselectively addressable functionalized template (RAFT) scaffolds to generate a Tn-clustered vaccine candidate.[337] They later introduced the first fully synthetic four-component glycolipopeptide vaccine PAM-$OVA_{257\text{-}264}$-PADRE-RAFT(α-GalNAc)$_4$ containing a clustered Tn B-cell epitope, a CD4+ Th epitope, a CD8+ CTL peptide epitope, and a palmitic acid that serves as a built-in adjuvant (Figure 22b6).[338] This vaccine successfully elicited strong Tn-specific IgG/IgM antibodies. The built-in PADRE and $OVA_{257\text{-}264}$ epitopes induced specific CD4+ and CD8+ T-cell responses, respectively, thus highlighting the correct antigen processing and presentation of both Th and CTL epitopes. In addition, the vaccine showed an exceptional prophylactic effect. No tumor was developed in any of the vaccinated mice with murine melanoma MO5 tumor cell challenge over 90 days.

7.3.2.2. MUC1 Glycopeptides

Strategies for active cancer immunotherapy based on synthetic MUC1-based glycopeptides have been addressed by impressive studies by Kunz and co-workers[141d,339] and others[340] Recently, Boons and co-workers introduced a new, three-component, fully synthetic vaccine containing a glycoundecapeptide with the tandem repeat unit of MUC1 bearing the monosaccharide Tn side chain, PV Th epitope, and a toll-like receptor 2 (TLR2) agonist (Pam_2CysSK_4 or Pam_3CysSK_4) that serves as the built-in adjuvant (Figure 22b5).[341] The lipid moiety of the vaccine facilitates the liposomal delivery, which assists multivalent antigen presentation, enhanced the uptake by APCs, and led to both T- and B-cell activation.[342] This vaccine induced exceptionally high titers of anti-MUC1 IgG antibodies, which could recognize MUC1-expressing cancer cells. In addition, low titers of antibodies induced against the Th epitope indicated that this self-adjuvanting, multicompnent vaccine setting could sup-

press anticarrier immune responses. Inspired by these results, Kunz and co-workers introduced a fully synthetic vaccine consisting of tumor-associated MUC1 glycopeptides and a lipopeptide ligand of the TLR2.[343] Although the antibody titers induced is not as high as those in the corresponding MUC1 TT vaccine,[344] the synthesis of this conjugate through fragment condensation with unprotected amino-functionalized glycopeptides can be applied to synthesize glycopeptides bearing sialic acid. Recently, Payne and co-workers successfully synthesized the first vaccine containing the TLR2 ligand $Pam_3CysSer$ and a full copy of the 20 amino acid MUC1 peptide VNTR domain, wherein all five *O*-glycosylation sites are derivatized with Tn and TF antigens.[345] Immunological evaluation of these constructs is currently underway.

7.3.3. Multivalent Vaccine Candidates

As transformed cells can have a distinct diversity of TACAs at their cell surface and their variety and quantity may vary at different stages of cellular development, it is desirable to design multiantigenic cancer vaccines to be closely associated with a particular cancer type.[346] One implementation from this idea is the polyvalent vaccine involving the administration of a mixture of monomeric conjugate vaccines.[347] Another attractive alternative arises from a unimolecular pentavalent construct consisting of different tumor antigens displayed on a single molecule (often a peptide) that would undergo a single conjugation step with a carrier protein or other platform for immunication. The KLH conjugates of Tn, Le^y, and globo H, which were found to elicit both IgM and IgG antibodies in murine hosts, have served as the proof of the principle of this approach.[348] Recently, Danishefsky and co-workers described the preparation and biological evaluation of a highly elaborate unimolecular pentavalent construct KLH conjugate of globo H, GM2, sTn, Tn, and TF (Figure 22 a3) in mice.[349] With an improved bioconjugation protocol, this vaccine candidate showed great promise in inducing both IgG and IgM antibodies against each of the five individual carbohydrate antigens. Another KLH conjugate of Gb3 with the peptide MUC5 Ac presented in a cluster form was also reported recently to target ovarian cancer.[350] This vaccine is expected to induce a strong immune response against both Gb3 and MUC5Ac. The immunological evaluation is currently underway.

8. Summary and Outlook

Significant progress in glycobiology and glycomedicine has driven the enormous development in oligosaccharide synthesis. Recent advances in synthetic methods as summarized herein, including programmable one-pot glycosylation and automated solid-phase synthesis, have allowed the development of convenient and effective automated system for the synthesis of oligosaccharide and glycoconjugates. However, many technical problems that hinder the development of carbohydrate research still remain. For example, laborious preparation of the building blocks is still necessary and unavoidable. Thus, it is valuable to conduct methodological investigation to expedite this process. For example, the innovation of the regioselective one-pot protection allows differentiation of sugar polyols in a single flask. Moreover, chemoenzymatic synthesis, which relies upon the regio- and stereoselective glycosylation of chemically derived substrates by enzymes (including glycosyltransferase, glycosidases, and glycosynthases), provides an opportunity to develop parallel and combinatorial synthesis of natural or non-natural carbohydrates of biological importance. Advances in the synthesis of complex carbohydrates will also facilitate the development of new methods for the synthesis of homogeneous glycoproteins.[154b] Indeed, advances in glycoscience depend heavily on structurally defined synthetic carbohydrates, which have proven to be valuable assets for developing glycoarrays and glycovaccines. In the future, it is expected that automated carbohydrate synthesis will be realized and readily accessible to provide various structures for biological studies.

Abbreviations

Ac	acetyl
AcP	acetyl phosphate
Ada	admantanyl
Alloc	allyloxycarbonyl
BDMS	bromodimethylsulfonium bromide
Bn	benzyl
Boc	*tert*-butoxycarbonyl
BOM	benzyloxymethyl
BSP	1-benzenesulfinylpiperidine
Bz	benzoyl
Cbz	benzyloxycarbonyl
ClAc	chloroacetyl
ClBn	*ortho*-chlorobenzyl
CMP	cytidine monophosphate
CSA	camphorsulfonic acid
DCC	*N*,*N*′-dicyclohexylcarbodiimide
DDQ	2,3-dichloro-5,6-dicyanobenzoquinone
DMAP	4-dimethylaminopyridine
DMF	*N*,*N*-dimethylformamide
DMTST	dimethyl(methylthio)sulfonium triflate
Fmoc	9-fluorenylmethoxycarbonyl
GDP	guanosine diphosphate
HPLC	high-performance liquid chromatography
*i*Pr	isopropyl
Lev	levulinoyl
M.S.	molecular sieves
2-NAP	2-naphthylmethyl
NBz	*para*-nitrobenzoyl
NIS	*N*-iodosuccinimide
Phth	phthalimido
Piv	pivaloyl
PMB	*para*-methoxybenzyl
PMP	*para*-methoxyphenyl
SBox	*S*-benzoxazolyl
TBAF	tetrabutylammonium fluoride
TBDPS	*tert*-butyldiphenylsilyl
TBS	*tert*-butyldimethylsilyl

TCA	trichloroacetyl
Tf	trifluoromethanesulfonyl
TFA	trifluoroacetyl
THF	tetrahydrofuran
TMS	trimethylsilyl
TMSOTf	trimethylsilyl trifluoromethanesulfonate
Tol	toluene
Troc	2,2,2-trichloroethoxycarbonyl
Tr	trityl
UDP	uridine diphosphate

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