Chapter 2

Chemical Synthesis of Carbohydrates and Their Surface Immobilization: A Brief Introduction

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Abstract

For all carbohydrate microarrays, two important prerequisites are necessary: the carbohydrate of interest has to be obtained either by isolation from natural sources, enzymatic or chemical synthesis; an immobilization of the carbohydrate at the surface of the chip has to be achieved. This chapter provides a very brief overview of the chemical synthesis of carbohydrates (creation of building blocks, assembly, and deprotection) and of immobilization techniques. Numerous methods are known to construct oligosaccharides by chemical methods. A typical monosaccharide building block, used in oligosaccharide assembly, is equipped with different protecting groups that mask the hydroxyl and amine groups. In general, a good leaving group at the anomeric center that can easily be activated is mandatory; especially trichloroacetimidates, phosphates, and thioethers have been widely used for the creation of glycosidic bonds. After the complete assembly of the oligosaccharide, a global deprotection of all permanent protecting groups affords the desired target structure with free hydroxyl groups. Linkers, which were introduced during the synthesis, must often be modified at the end to create appropriate functionalities for surface immobilization.

Key words: Glycosylation, Protecting group, Deprotection, Building block, Chemical synthesis, Surface immobilization, Microarrays, Thiols

1. Introduction

The chemical synthesis of complex oligosaccharides has been a synthetically difficult and rather time-consuming endeavor (1). In contrast to peptides and oligonucleotides, carbohydrates are often branched and several hydroxyl or amino groups at each building block lead to a dazzling variety of structures, which can be obtained by a small set of subunits. Even more complicated is the creation of a new stereogenic center resulting from the formation of a glycosidic bond during the combination of two monosaccharides.

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Table 1
Number of different oligomers for nucleotides, peptides, and carbohydrates

	Numbers of different oligomers		
Oligomer size	**Nucleotides**	**Peptides**	**Carbohydrates**
1	4	20	20
2	16	400	1,360
3	64	8,000	126,080
4	256	160,000	13,495,040
5	1,024	3,200,000	1,569,745,920
6	4,096	64,000,000	192,780,943,360

The numbers for the carbohydrates are based on the "10 mammalian" monosaccharide units. Branching is also taken into account

A recent statistical analysis regarding the diversity of mammalian carbohydrate structures – based on the "10 mammalian monosaccharides" without considering any further attachments – reveals astounding numbers, which dwarf the complexity of both peptides and oligonucleotides (Table 1) (2). Although the number of structural combinations encountered in nature is much smaller than the theoretical ones, it is obvious that synthetic carbohydrate chemists have to face a variety of challenges during the assembly of an oligosaccharide by chemical means.

2. Chemical Synthesis of Carbohydrates

A carefully designed synthetic plan is necessary before one starts with the total synthesis of the desired target structure. Such a plan includes the glycosylation strategy (type of anomeric leaving groups) and some kind of temporary protecting groups (3, 4). After that, mono- or disaccharide building blocks have to be prepared. With these compounds in hand, the assembly process can be started. Besides well-known solution-phase techniques, an automated solid-phase approach has also been developed (5, 6). To gain access to the native structure, a deprotection is required. Before the carbohydrate of interest is attached to the surface of a microarray other required steps include the functionalization of the linker, purification steps (such as dialysis or the use of HPLC), and quality control (mass spectrometry and NMR analyses). A simplified scheme of the different steps from the desired target structure to surface immobilization is depicted in Fig. 1.

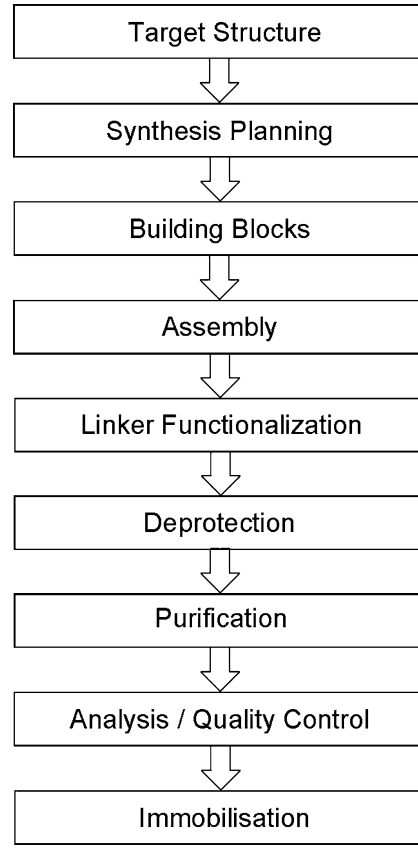


Fig. 1. Required steps from defining the carbohydrate target structure until the immobilization of the synthesized oligosaccharide.

2.1. Building Blocks

A typical monosaccharide building block used in oligosaccharide assembly is equipped with different protecting groups that mask the hydroxyl and amine groups. Two different kinds of protecting groups, permanent and temporary ones, need to be distinguished. The former mask hydroxyl moieties have to be unveiled at the end of the synthesis whereas the latter mark sites of further glycosylation. Permanent protecting groups are commonly ethers (mostly Bn ethers), which can be cleaved by reduction at the end of the synthesis, but rather stable esters (such as Piv or even Bz esters) are also used. Temporary protecting groups are functional groups that are easy to cleave (but should be stable during the glycosylation reaction) such as trityl ethers, fluorenylmethoxycarbonyl (Fmoc) groups, and several silyl groups, while acetates (Ac) are often employed as temporary protecting groups. However, whether a protecting group is used as permanent or temporary is often depend strongly on the glycosylation strategy (3). Besides controlling regioselectivity by orthogonal protecting groups to account for possible branching of the carbohydrate chain, the stereochemistry

at the anomeric carbon must be controlled during the formation of the glycosidic bond. Placement of participating protective groups at the C-2 hydroxyl or amine groups ensures the formation of *trans*-glycosidic linkages. In contrast, nonparticipating groups and low temperatures during the glycosylation lead to the preferential installation of *cis*-glycosides.

Furthermore, an anomeric leaving group that can be easily activated to induce the formation of the glycosidic linkage is needed (3, 7). A large variety of anomeric leaving groups are known and several of them are widely utitized in complex oligosaccharide synthesis. Figure 2 depicts a selection of such anomeric leaving groups (**1**–**7**) with their respective activation procedures to perform a glycosylation reaction (3). The oldest anomeric leaving groups used for the chemical synthesis of saccharides are glycosyl bromides **1** that are activated by silver salts. Today, the most commonly used anomeric leaving groups are phosphates **2**, trichloroacetimidates **3**, and thioethers **4** (3).

To date, the synthesis of building blocks is the most time-consuming process of oligosaccharide synthesis. Rather than synthesizing each building block separately integrated synthetic paths that grant access to several building blocks from a common precursor are desirable.

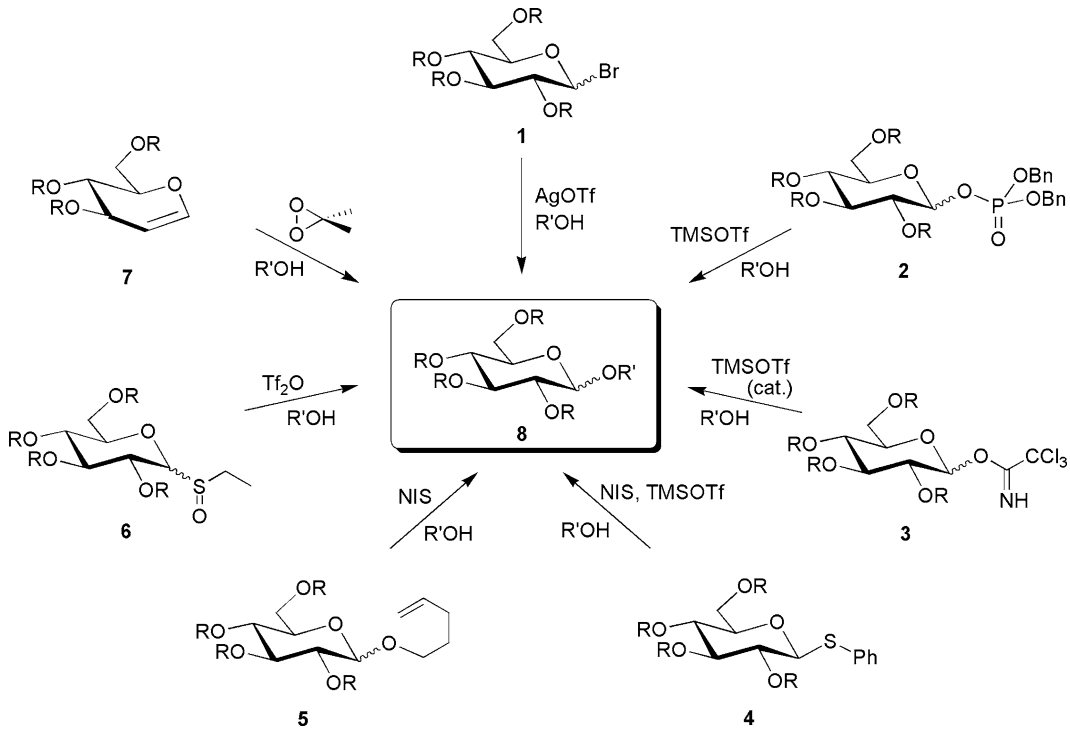


Fig. 2. Various anomeric leaving groups in **1**–**7** and their respective activation modes.

Commonly, the differently protected and functionalized monosaccharides are accessed from naturally occurring sugar starting materials through a series of protection–deprotection maneuvers. Such a process establishes the desired protecting group pattern and typically requires 6–20 steps depending on the sugar, the protecting group ensemble, and the anomeric leaving group.

A plethora of different methods to chemically distinguish the different hydroxyls already exists. The reactivity of the anomeric hydroxyl group that is part of a hemiacetal differs significantly from that of the other hydroxyls. The selective removal of acetate esters in the anomeric position and the readily occurring substitution of esters by alcohols such as *n*-pentenol or *p*-methoxybenzyl alcohol in the presence of Lewis acids are well established (8, 9). The C-6 hydroxyl also exhibits special reactivity as the only primary hydroxyl group of the sugar moiety. In addition, the C-6 hydroxyl is sterically exposed and increased reactivity often leads to high regioselectivity (9).

Another very popular method for the selective protection of four or five hydroxyls relies on rendering two of them silent. *cis*-Hydroxyls easily form 5-membered rings, so-called isopropylidenes (9), upon treatment with acetone dimethylacetal in the presence of a Lewis acid (Fig. 3). Benzaldehyde and benzaldehyde dimethylacetal are prone to form 6-membered rings (benzylidene acetals, **10**) involving the C-4 and the C-6 hydroxyl. An elegant method to lock the C-1 and C-2 hydroxyls is the formation of an orthoester **11** (Fig. 3). Usually orthoesters are synthesized by nucleophilic

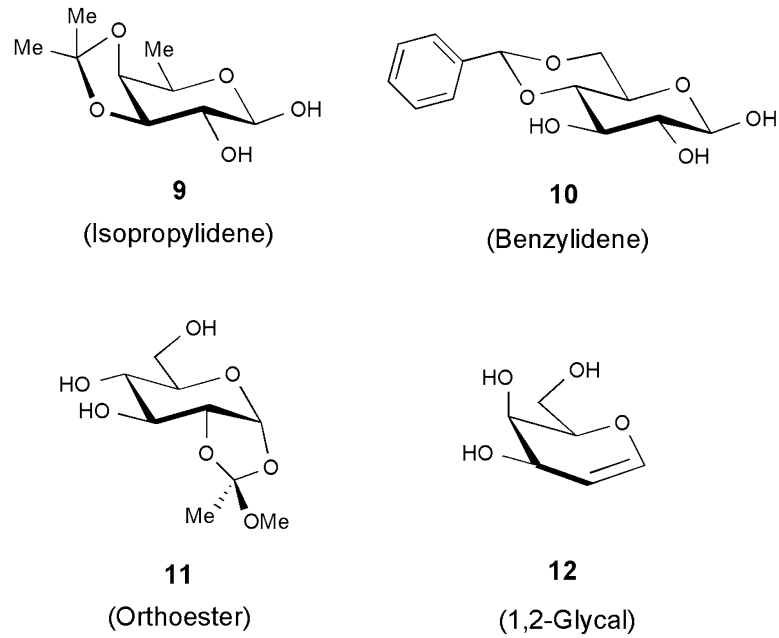


Fig. 3. Methods used in building block synthesis to render two hydroxyls "silent".

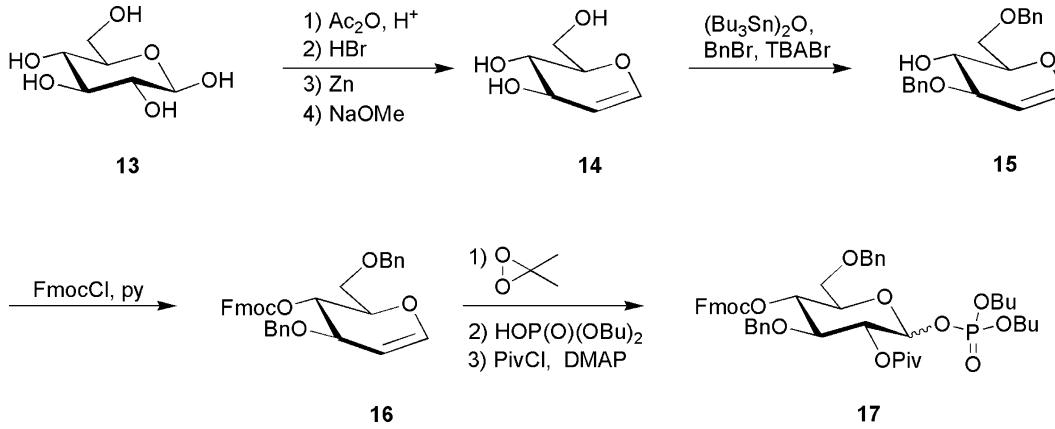


Fig. 4. Synthesis of glycosyl phosphate **17** starting from D-glucose (**13**).

attack of an alcohol at the oxocarbenium ion that can be generated by a glycosyl bromide with the neighboring ester group in the presence of a nonnucleophilic base (9). This method commonly yields two stereoisomeric products. The elimination of two hydroxyls to form a 1,2 double bond is yet another method to reduce the number of hydroxyl groups to be distinguished. The remaining hydroxyls of these 1,2-glycals **12** are more easily distinguished. Finally, selective oxidation procedures allow the reinstallation of the eliminated hydroxyls at a later stage (9).

For example, Fig. 4 shows the generation of a glucosyl phosphate with Fmoc as a temporary protecting group in position 4. D-Glucose is used as starting material. After peracetylation and anomeric substitution of acetate by bromide, Zn-mediated reduction eliminates two hydroxyl groups of the glucose **13** to afford the glucal **14**. Protection with permanent protecting groups (Bn) in positions 4 and 6 mediated by a tin reagent and further protection with FmocCl as temporary protecting group yielded **16**. Epoxidation with dimethyl dioxirane (DMDO) is followed by opening of the 1,2-anhydrosugar with dibutylphosphate. Protection of the ensuing C2 hydroxyl group with a participating group (e.g. pivaloyl chloride [PivCl)] produced the desired glucosyl phosphate **17** in excellent yield (10, 11).

2.2. Glycosylation Reaction and Assembly

Definitely it would go beyond the scope of this brief chapter to discuss in detail all the parameters which affect the glycosylation reaction (3, 4, 12). However, a short overview of the mechanistic picture of this important reaction seems to be appropriate. Nevertheless, detailed information about glycosylation mechanisms is mostly fragmented. Commonly, the glycosylation reaction involves nucleophilic displacement at the anomeric carbon. Due to the endocyclic oxygen next to the anomeric center a positive charge

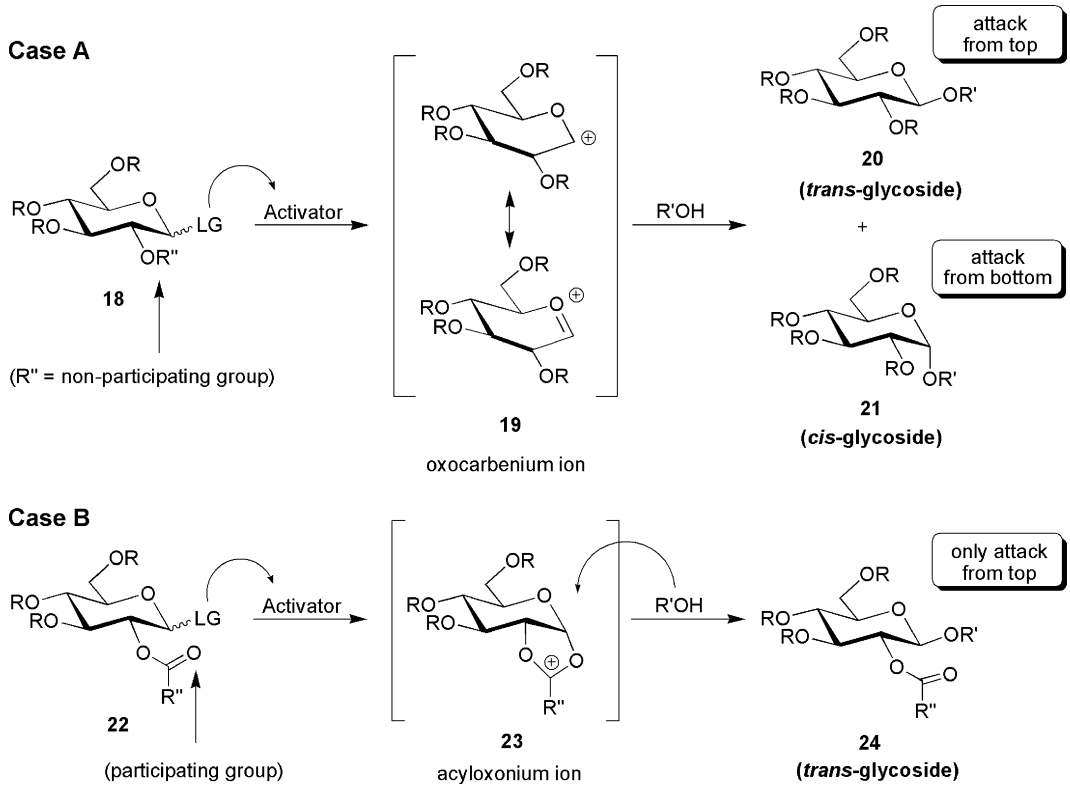


Fig. 5. Simplified mechanistic picture of a glycosylation reaction either with non-participating group (Case A) or with participating group (Case B) in position 2.

is strongly stabilized. Thus, after activation of **18** one may consider a glycosyl cation **19** (oxocarbenium ion) as intermediate if no further stabilising substituents are in reach (Fig. 5, Case A). A nucleophile such as a hydroxyl group can attack this species either from the top or from the bottom face; a mixture of *trans*- and *cis*-glycosides **20** and **21** results. In the case of a participating group in position 2, such a carbocation might also be stabilized by neighboring group participation leading to an acyloxonium ion as major intermediate (Fig. 5, Case B). Such an acyloxonium ion **23** shields either the top or the bottom face (depending on the stereochemistry at C-2). As a result, the attack of the nucleophilic hydroxyl group can only take place from the nonshielded side leading to *trans*-glycoside **24** as major isomer. Using this model, it is easy to understand why participating groups must not be used for the formation of *cis*-glycosides. For the creation of *cis*-glycosides, one has to rely, in most cases, on thermodynamic considerations. In the case of glucose, galactose, and fucose, as well as in their corresponding amines and acids, the α product is thermodynamically preferred due to the anomeric effect. Low reaction temperatures favor their formation. However, a complete α selectivity is often difficult to

achieve. In the case of mannose, the *cis*-mannosides are the β product. Therefore, the creation of β-mannosides requires other approaches that have been described in the literature (12, 13).

In addition to the glycosylation reaction, the second important reaction in oligosaccharide assembly is the highly selective removal of protecting groups (9). For branched oligosaccharides, careful synthetic planning with respect to the temporary protecting group ensemble is required. Permanent protecting groups stay unaffected until the very final steps of the synthesis to procure the completely deprotected carbohydrate. The cleavage of temporary protecting groups after each glycosylation reaction is essential to liberate hydroxyls that are needed as nucleophiles for the next glycosylation step. Silyl protecting groups (e.g. in **25**) are cleaved by fluoride sources or strong acids whereas fluorenylmethoxycarbonyl (Fmoc) groups (e.g. in **27**) are cleaved by weak bases such as piperidine. Stronger bases such as sodium methoxide are required for the clevage of acetate (Ac) groups (as shown for **29**) whereas levulinoate (Lev) esters in **31** need only hydrazine to be cleaved. An overview of four deprotection methods is provided in Fig. 6. Caution has to be taken by employing two temporary protecting groups next to each other. In some instances, a migration of the remaining protecting group might occur when one of the two is removed (especially in the presence of bases) (8).

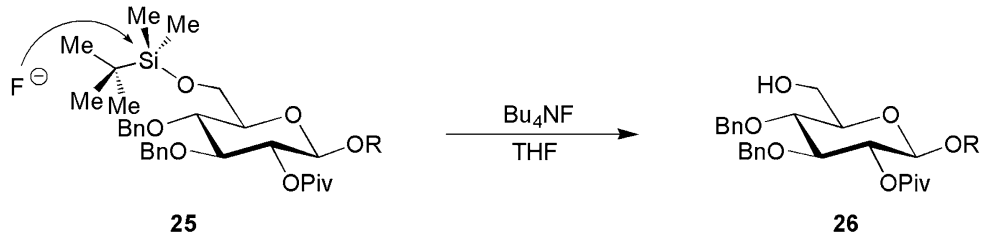
2.3. Global Deprotection

Once the oligosaccharide is assembled by solution- or solid-phase techniques, the deprotection of all permanent protecting groups (global deprotection) is a challenging endeavour. Two approaches dominate: The first is a single-step procedure with sodium, carried out in liquid ammonia, often referred as Birch reduction (8).

Benzyl ether groups as well as esters and carbonates are removed. The fact that olefinic moieties (except allyl) stay unaffected is the major advantage since terminal CC double bonds are often used as a chemical handle, as a future point of attachment to the surface of a microarray or to a carrier protein (8, 14). Thus it should not be attacked during the deprotection sequence. Besides olefinic moieties, the reducing end hemiacetal of an oligosaccharide is also maintainable during Birch debenzylation (15). Sometimes the commonly employed trichloroacetyl (TCA) amino protecting group causes problems resulting in a tremendous reduction of yield. To circumvent these difficulties, the transformation of the TCA group into an acetyl group by a radical initiated reduction with Bu_3SnH is advantageous. To facilitate the further purification process, the unprotected sugar, obtained by Birch reduction, is frequently peracetylated using acetic anhydride and pyridine (9, 10).

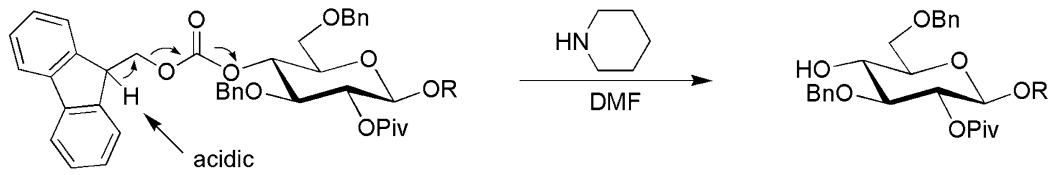
The experimental setup for the Birch reduction using sodium in liquid ammonia is relatively high. Minor impurities in the starting material may cause a serious decrease in yield. A major advantage compared to hydrogenolysis procedures is avoidance of heavy

Silyl Groups (*e.g.* TBS)



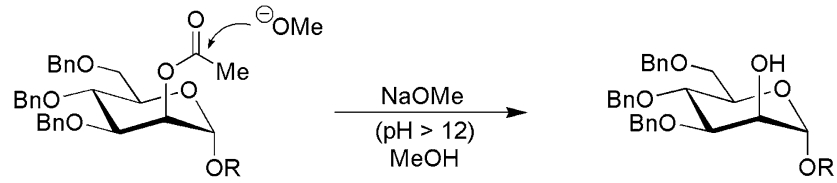
25 **26**

Fluorenylmethoxycarbonyl (Fmoc) Groups



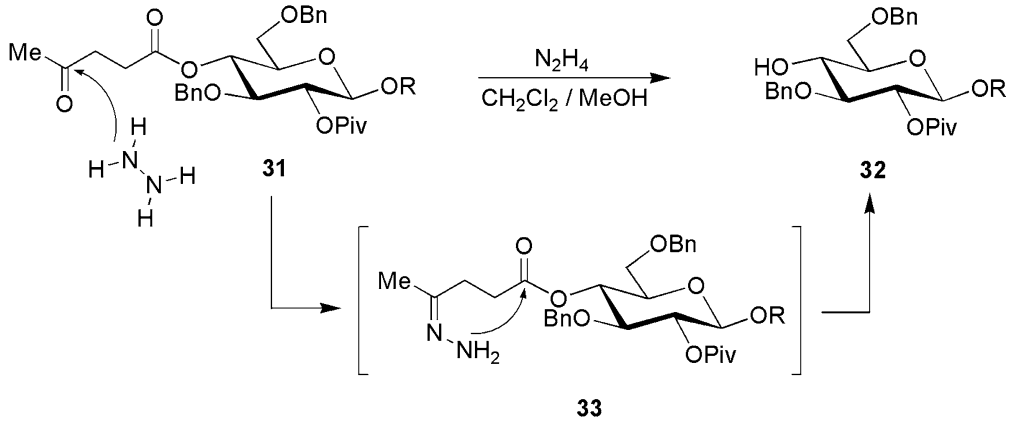
27 **28**

Acetate (Ac) Groups



29 **30**

Levulinoyl (Lev) Groups



31 **32** **33**

Fig. 6. Four examples of temporary protecting groups (TBS, Fmoc, Ac, and Lev) and methods for their selective removal.

metals. Traces of such metals may cause problems in biological experiments employing the synthetic carbohydrates.

The other protocol requires two steps. In a first step, ester groups have to be removed by base, before hydrogenolysis cleaves the benzyl (Bn) ethers (9). In the latter case, palladium on charcoal or $Pd(OH)_2$ on charcoal (Pearlman's catalyst) are widely used as catalysts. The reaction itself takes place under an atmosphere of hydrogen. Commonly the reaction proceeds well and is finished over night. If problems with the deprotection of several benzyl ethers (e.g. sterically very hindered Bn groups) occur, hydrogen pressure up to 100 bar may be applied. It is evident that the conditions employed for hydrogenolysis also reduce carbon–carbon double bonds. If one needs an alkene as a handle, the Birch reduction is the method of choice for global deprotection.

2.4. Case Study of Blood Group A Antigen

To illustrate the process of carbohydrate assembly, the blood group A antigen **34** with a thiol linker is chosen as an example (16). This glycan is a branched tetrasaccharide consisting of galactosamine, fucose, galactose, and glucosamine (Fig. 7). For the installation of a chemical handle being necessary for surface immobilization, a pentenyl group was attached to the reducing end of the sugar. Such an olefinic moiety can be transformed easily at the end of the total synthesis into a thiol unit. Figure 7 shows the retrosynthetic analysis into five building blocks **36**–**40**. Pivaloyl (Piv) and acetate (Ac) esters are employed as permanent protecting groups for hydroxyls benzyl (Bn) ethers. The amine moieties are protected in the case of galactosamine **36** as azide (N_3), in the case of glucosamine **40** as trichloroacetamide (TCANH). In the latter case a participating group, namely TCA, is necessary to achieve β selectivity whereas nonparticipating nitrogen functionality, namely the azide, is necessary to achieve α selectivity. For the branching galactose **37**, two orthogonal temporary protecting groups are required. Fmoc in position 2 ensures, with its ability for neighboring group-participation, β selectivity and can be cleaved later on by the weak base piperidine without affecting the levulinoate in position 3. In the case of the fucose building block **39**, it is again important to have a nonparticipating group in position 2 to afford mainly the α product. For the anomeric leaving groups, a mixed strategy consisting of one trichloroacetimidate and three phosphates was chosen. This selection was based on arguments with respect to the ease of building block synthesis.

Figure 8 summarizes the assembly and deprotection process (16). The pentenyl functionalized monosaccharide **41** and galactosyl phosphate **37** were reacted in the presence of the Lewis acid TMSOTf, as activating agent, to produce a disaccharide whose Fmoc group was immediately deprotected to afford **42**. Further fucosylation and removal of levulinoate in the presence of hydrazine yielded trisaccharide **43**. To achieve high α selectivity,

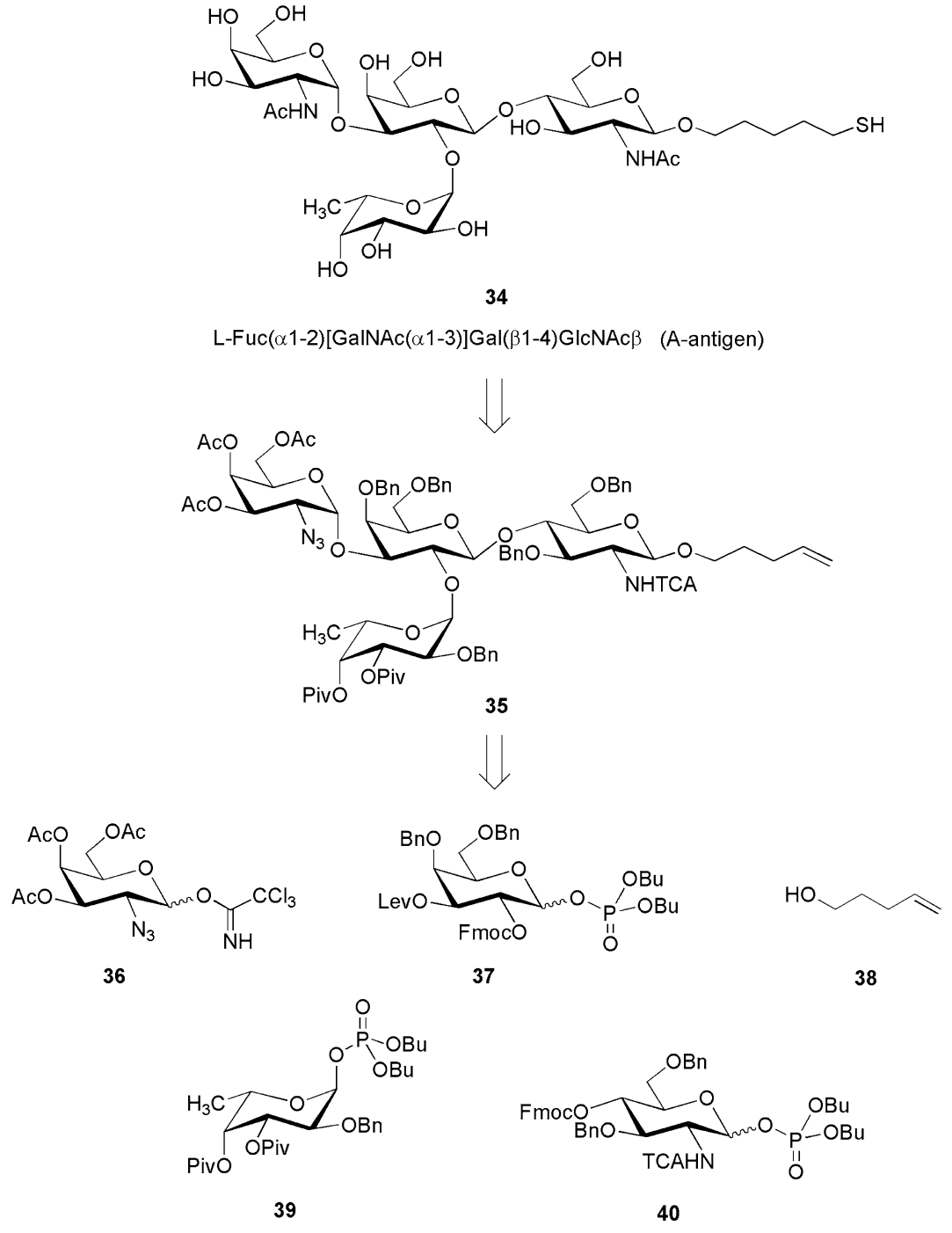


Fig. 7. Retrosynthesis of the A antigen **34** with a thiol linker, building blocks **36–40**.

the last glycosylation was carried out at low temperatures. The completely protected tetrasaccharide **35** was subjected to Birch reduction. Sodium in liquid ammonia removed all permanent protecting groups and transformed the azide moiety as well as the

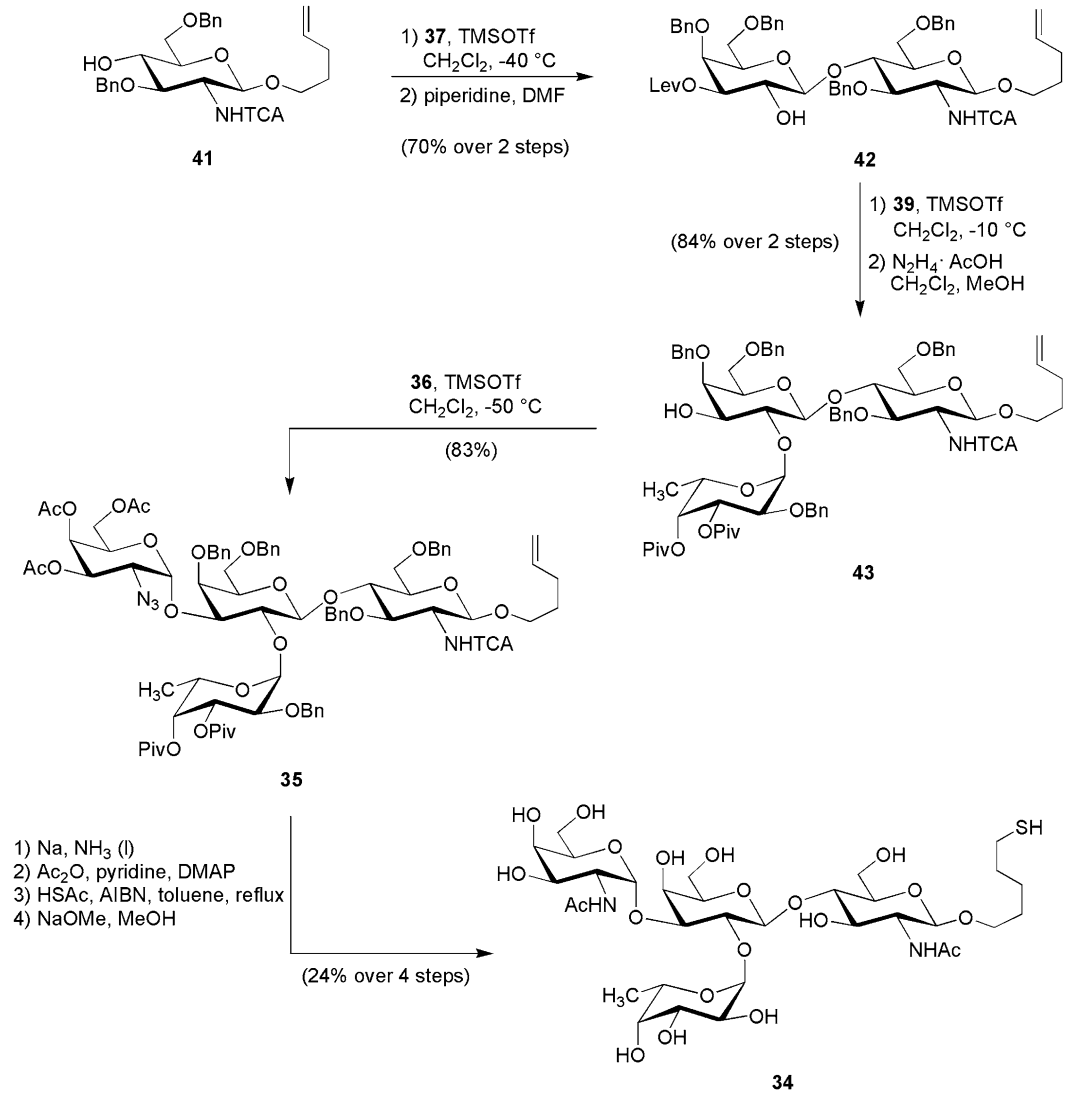


Fig. 8. Assembly of the tetrasaccharidic A antigen **34** with a thiol linker.

trichloroacetamide into "naked" amines, thus achieving global deprotection. The reaction with acetic anhydride and pyridine converted all hydroxyls to the corresponding acetates and all amines to the respective acetamido groups. This product is much less polar than the compound directly obtained after Birch reduction and can be more easily purified. A radical-initiated addition of thioacetic acid introduced the sulfur moiety (16). In the final step all acetates are cleaved to generate the native structure **34**. Dialysis might be a good choice to get rid of salts, which might have accumulated during the last steps of the synthesis. Lyophilisation yields the carbohydrate ready for attachment to microarrays.

3. Immobilization Techniques

Microarrays in the "chip" format, prepared by attachment of biopolymers to a surface in a spatially discrete pattern, have enabled a low-cost and high-throughput methodology for screening interactions involving these molecules (17–19). The most important advantages compared to classical methods are that microarrays allow for several thousand binding events to be screened in parallel, hence the experiment requires only miniscule amounts of both analyte and ligand. Thus, binding profiles and lead structures can be readily examined. In addition, carbohydrate microarrays are ideal to detect interactions that involve carbohydrates since the multivalent display of ligands on a surface (cluster effect) overcomes the relative weakness of these interactions by mimicking cell–cell interfaces. However, an important question to be tackled is the way in which a carbohydrate becomes immobilized on the surface. This book provides and discusses a large variety of methods in detail. However, in the following paragraphs I would like to provide an overview of some selected techniques from the viewpoint of an organic chemist.

Older methods for the preparation of carbohydrate microarrays consist of nitrocellulose-coated slides (in the case of noncovalent immobilization of microbial polysaccharides) (20) or self-assembled monolayers modified by Diels–Alder mediated coupling of cyclopentadiene-derived oligosaccharides (21). Unfortunately, the former method requires large polysaccharides or lipid modified sugars for the noncovalent interaction. The latter requires the preparation of oligosaccharides bearing the sensitive cyclopentadiene moiety. Today noncovalent immobilization is mainly based on fluorine–fluorine interactions (22) or DNA-directed immobilization (23). In the first case, the surface is coated by perfluorinated hydrocarbons and the carbohydrate to be attached bears a perfluorinated alkyl chain. In the latter case, the specific pairing of DNA bases due to the different number of hydrogen bridges is the key to immobilization; one single strand of DNA is attached to the carbohydrate moiety whereas the complementary one is attached to the surface of the microarray.

For covalent immobilization (24–26) very good results were obtained by utilizing maleimide functionalization of glass slides and the immobilization of the oligosaccharides with thiol-containing linkers **44**. As pointed out in the Subheading 2.4, thiols are obtained in a facile way by reacting double bonds with thiolacetic acid under radical conditions (either initiated by light or radical starters such as AIBN). Sodium methoxide or even potassium carbonate cleaves the thioacetate to generate the free thiol. In a Michael-type addition the thiol moiety attacks the maleimide functionality. A stable covalent bond is formed to afford modified surfaces of type **45**. Care has to be taken when thiol-containing substrates are

stored for a longer time. Oxygen from the air leads to the formation of disulfide linkages that no longer react with maleimides. Therefore, a weak reducing agent is often added to generate the thiols out of the respective disulfides. Another possible reaction partner is iodoacetyl groups, which are immobilized on a surface. Due to the high nucleophilicity of the sulfur, a simple nucleophilic substitution leads to immobilized saccharides of type **46** (Fig. 9).

Also, other moieties such as linkers functionalized with azides are utilized for surface immobilization. In a Huisgen 1,3-dipolar cycloaddition, a five-membered trizole is formed with an alkyne under the catalytic influence of copper. This chemistry is often referred to today as "click-chemistry" (27). Of course, the carbohydrate part can bare an alkyne unit, while the surface bares the respective azide. Also, primary amines, which react under conditions of a reductive amination with aldehydes or vice versa to secondary amines, are utilized. However, the generation of aldehydes – either as part of a linker attached to the carbohydrate moiety or as part of the surface – is difficult, and decomposition may occur readily. If amines are used as reactive group for immobilization, one should use an activated amide as counterpart. Both functionalities are rather stable, and high yields are obtained. If one likes to use thiols instead of amines, but due to synthetic considerations amines have

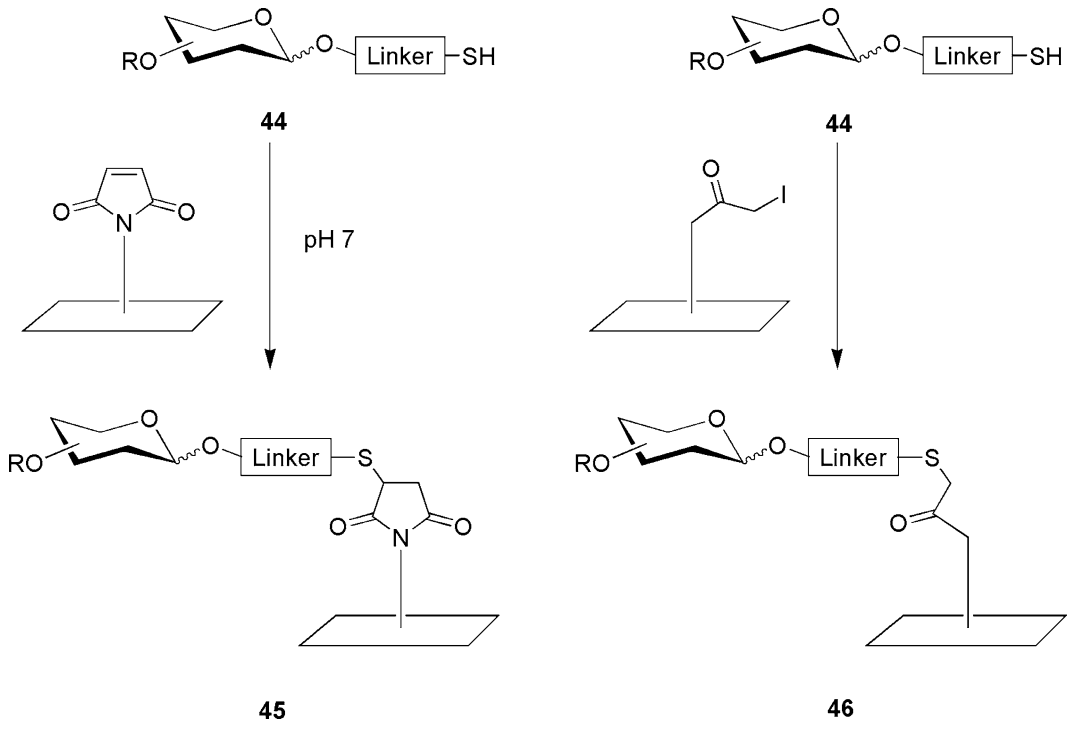


Fig. 9. Two possibilities to attach thiol-functionalized saccharides to surfaces.

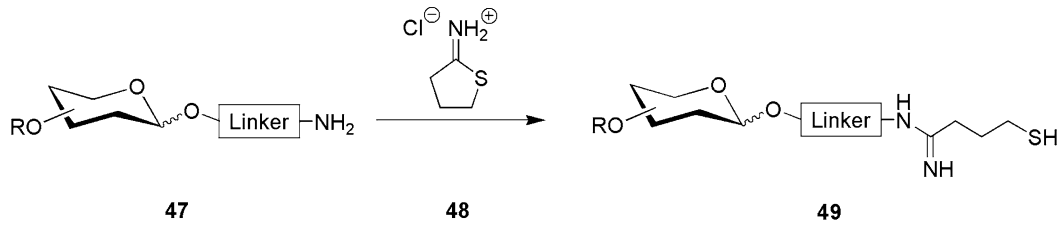


Fig. 10. Transformation of an amine-functionalized linker **47** into a thiol-functionalized linker **49** by using Traut's reagent **48**.

been created, Traut's reagent **48** will be able to convert amines **47** into corresponding thiols **49** (Fig. 10) (28). By this procedure, the length of the linker is increased by five heavy atoms.

In general, every simple reaction that is easy to handle and promises a high reaction yield can be used for the immobilization of carbohydrates. The bonds formed have to be stable and should not be cleaved easily by hydrolysis. Of course, the corresponding functional groups have to be installed on both reaction partners and the oligosaccharide as well as the modified surface of the microarray. Further considerations that should be taken into account are the kind of linker, its length, and the point of attachment. Polyethylenglycol (PEG) linkers reveal a higher degree of water solubility than common alkyl chains. Linkers that are too short might cause problems when large proteins interact with the carbohydrate. As point of attachment, in most cases, the anomeric center is chosen to mimic the native structure; however, of course, other functionalization sites are also possible.

4. Conclusion

Carbohydrate synthesis is still a rather time-consuming endeavour; however, major efforts have been done during the last decades to fasten this process such as the use of integrated paths to create building blocks, improved glycosylation strategies and even the introduction of automated methods for the assembly of oligosaccharides. The right choice of temporary and permanent protecting groups, participating and nonparticipating ones, is the prerequisite for a successful stereoselective assembly. As anomeric leaving groups especially trichloroacetimidates, phosphates, and thioethers have become prominent. In a final step, global deprotection has to be performed, either by saponification and hydrogenolysis or by Birch reduction. For surface immobilization, handles such as pentenyl chains can be easily converted at the end of the synthesis into thiols.

Acknowledgments

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