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Automated Synthesis of the Tumor-Associated Carbohydrate Antigens Gb-3 and Globo-H: Incorporation of α-Galactosidic Linkages

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Carbohydrates are displayed on the surface of both normal and tumor cells as glycosphingolipids (GSLs), glycoproteins, and GPI anchors. Cancer cells express altered cell surface glycoconjugates, and some GSLs act as adhesion molecules during tumor cell metastasis.[1]

The GSLs Globo-H (**1**) and Gb-3 (**2**) (Scheme 1) have been identified as antigens of a variety of different cancer types. Globo-H (**1**) is currently being evaluated in clinical trials as an anti-tumor vaccine for the treatment of breast and prostate cancers.[2,3] The trisaccharide glycolipid Gb-3 (**2**) is a receptor for Shiga-like toxins[4] and has recently been implicated in the entry of HIV-1 into cells.[5] Due to their biological importance, these antigens have been the subject of several total syntheses that showcased different methods.[3,6−14]

The Globo series of carbohydrate antigens require the selective installation of a *cis*-galactosidic linkage on the axial C4 hydroxyl of galactose (Scheme 1). The stereochemical outcome of *cis*-glycoside formation cannot be controlled via a C2 participating group. α-Galactosidic linkages have been installed in solution-phase chemistry using a variety of glycosylating agents.[15]

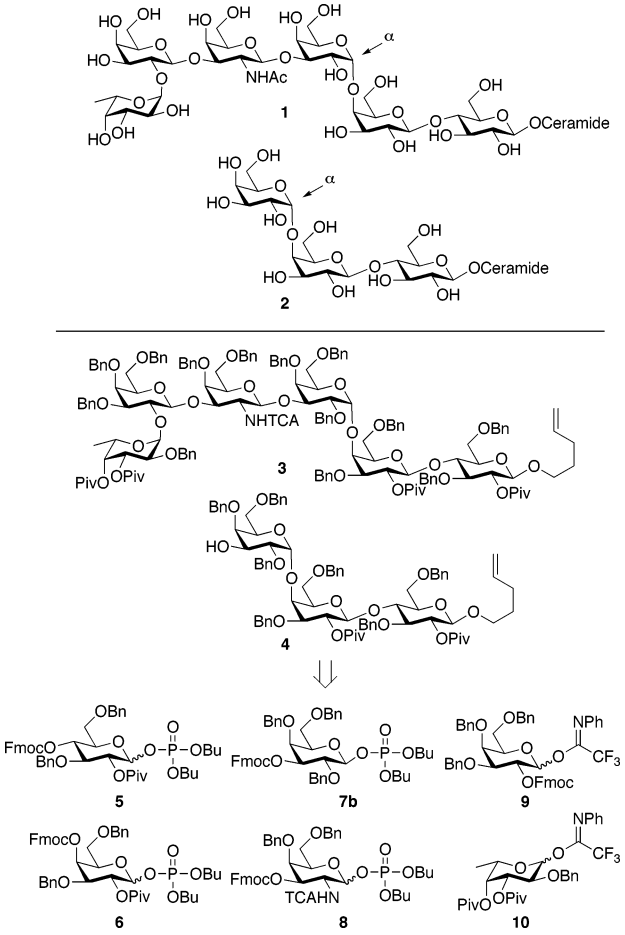
Our initial work on automated oligosaccharide synthesis demonstrated that linear as well as branched carbohydrates can be assembled.[16,18] However, the creation of challenging *cis*-glycosidic linkages such as α-galactosides had not been achieved by automated synthesis.

Here, we describe the automated solid-phase assembly of the protected tumor-associated oligosaccharide antigens Gb-3 (**4**) and Globo-H (**3**). Six building blocks (**5**−**10**) are required for the fully protected Globo-H hexasaccharide **3** (Scheme 1). Each monomer, except the final fucose moiety **10**, contains a temporary protecting group. We chose fluorenylmethoxycarbonyl (Fmoc) that is completely stable under the acidic glycosylation conditions and is readily cleaved by a weak base such as piperidine for temporary protection.[17] The analysis of the Fmoc deprotecting solution provides a quantitative assay for the efficiency of each glycosylation/deprotection cycle during automated assembly.[18]

Installation of the α-galactosidic linkage proved to be crucial for the assembly of Gb-3 trisaccharide **4** in anticipation of the synthesis of the larger oligosaccharide **3**. High α-selectivity is mandatory for the coupling since the solid-phase approach allows for purification only after completion of the synthesis. Investigations aimed at optimizing coupling efficiency and selectivity were performed by automated solid-phase synthesis and are summarized in Table 1. Resin-bound lactose acceptor **11** was assembled by automation using standard building blocks **5** and **6** as detailed in Scheme 2. Support-bound disaccharide **11** was then glycosylated using different galactose building blocks. Coupling efficiency and selectivity were rapidly determined by LC−MS analysis of the cleaved products.

The α- and *β*-glycosyl phosphates containing a C2 participating group differ only in the reaction kinetics.[19] In contrast, the two

Scheme 1. Tumor-Associated Carbohydrate Antigens of the Globo Series **1** and **2** and Their Protected Forms **3** and **4** That Can Be Derived from Monosaccharide Building Blocks **5**−**10**



anomers of building block **7** showed strongly different selectivity (entries 1 and 2). The *β*-anomer **7b** resulted in a significantly better α/*β* ratio on solid support than the corresponding α-anomer **7a** (14:1 vs 4:1). Longer reaction times (3 h) and low temperatures (−50 °C) were required in order to drive the reaction to completion and to achieve the desired selectivity. The *β*-glycosyl trichloroacetimidate **12** showed similar selectivity (entry 3). The presence of a benzoate group in the C4 position (**13**) resulted in poor selectivity and byproduct formation due to migration of the benzoate group during Fmoc deprotection (entry 4). Protected Gb-3 was assembled in 12 h using building blocks **5**, **6**, and **12** (Scheme 2). Cleavage from the solid support by olefin cross-metathesis in the presence of Grubbs' catalyst and HPLC purification yielded pure **4** in 46% yield.[20]

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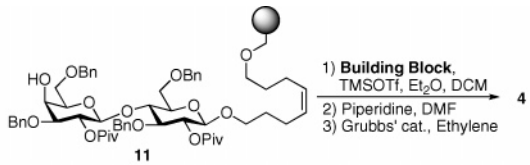
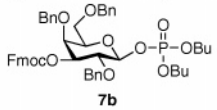
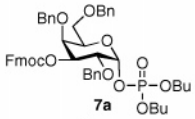
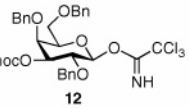
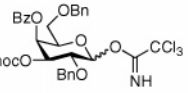
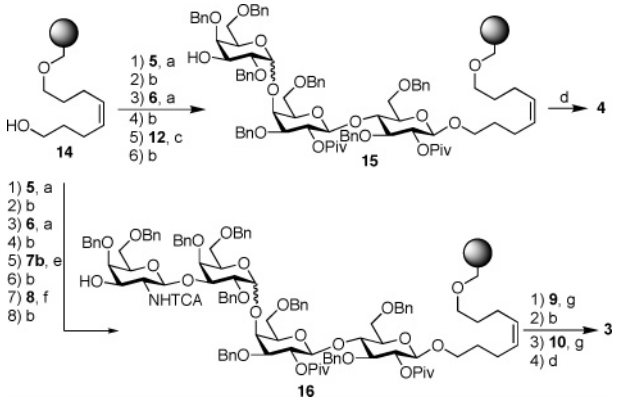
Table 1. Anomeric Selectivity of Gb-3 Using Different Building Blocks



entry	α-galactose building block	α:β selectivity of **4**[a]
1	 **7b**	14:1
2	 **7a**	4:1
3	 **12**	11:1
4	 **13**	4:1

[a] Determined by LC−MC of crude mixtures.

Scheme 2. Automated Synthesis of Trisaccharide **4** and Hexasaccharide **3**[a]



[a] Conditions: (a) building block (5 equiv), TMSOTf (5 equiv), DCM, −15 °C, repeated once for 45 min each; (b) piperidine (20% in 2 mL of DMF), repeated twice for 5 min each; (c) building block (5 equiv), TMSOTf (0.5 equiv), DCM, −30 °C, repeated once for 1 h each; (d) Grubbs' catalyst (first generation), ethylene atmosphere, $CHCl_2$, rt, overnight; (e) building block (5 equiv), TMSOTf (5 equiv), Et_2O, DCM, −50 °C, repeated once for 3 h each; (f) building block (3.3 equiv), TMSOTf (3.3 equiv), DCM, −15 °C, repeated twice for 25 min each; (g) building block (5 equiv), TMSOTf (0.5 equiv), DCM, −10 °C, repeated once for 25 min each.

After establishing a procedure to introduce α-galactosidic linkages with high selectivity, protected Globo-H hexasaccharide was prepared. Building blocks **5**, **6**, **7b**, and **8** were used to assemble the resin-bound tetrasaccharide **16**. This tetrasaccharide was unstable in the presence of stoichiometric amounts of Lewis acid necessary for the activation of glycosyl phosphate building blocks. Glycosyl *N*-phenyl trifluoroacetimidate building blocks **9** and **10** performed well under mild conditions.[21] Assembly of the hexasaccharide required 25 h before cleavage from the polymer support by olefin cross-metathesis yielded the crude product. LC−MS analysis of the crude mixture obtained by cleavage from the support after assembly was employed to assess the outcome of the automated synthesis (Figure 1). The desired product **3** (17.7 min) is the major product along with small amount of the β-anomer (17.2 min) and some deletion sequences. Careful purification by column chromatography afforded **3** in 30% overall yield from resin **16**. Cleavage of all protective groups on hexasaccharide **3** was performed under Birch conditions as established earlier.[14]

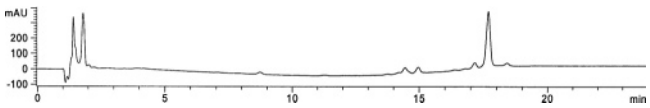


Figure 1. HPLC trace of crude, fully protected Globo-H (**3**) after automated synthesis and cleavage from solid support (UV absorbance at 209 nm).

In conclusion, the automated assembly of two tumor-associated carbohydrate antigens is reported. A solution for the construction of α-galactosidic linkages on solid support is presented in the context of linear syntheses of the complex oligosaccharides Globo-H and Gb-3. LC−MS analysis has become an important tool to monitor rapidly the success and selectivity of oligosaccharide syntheses. Research focusing on the construction of other challenging linkages such as β-mannosidic linkages and the incorporation of sialic acid into automated synthesis protocols is ongoing.

Acknowledgment. Funding from the ETH Zürich, the Swiss National Science Foundation (SNF Grant 200121-101593), the DFG (Emmy Noether Grant to D.B.W.), and the Alexander von Humboldt Foundation (Feodor Lynen Fellowship to D.B.W.) is gratefully acknowledged.

Supporting Information Available: Detailed procedures for automated assembly, analytical data, and spectra for the compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Anomeric Reactivity-Based One-Pot Synthesis of Heparin-Like Oligosaccharides

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Abstract: A highly efficient one-pot methodology is described for the synthesis of heparin and heparan sulfate oligosaccharides utilizing thioglycosides with well-defined reactivity as building blocks. L-Idopyranosyl and D-glucopyranosyl thioglycosides **5** and **10** were used as donors due to low reactivity of uronic acids as the glycosyl donors in the one-pot synthesis. The formation of uronic acids by a selective oxidation at C-6 was performed after assembly of the oligosaccharides. The efficiency of this programmable strategy with the flexibility for sulfate incorporation was demonstrated in the representative synthesis of disaccharides **17**, **18**, tetrasaccharide **23**, and pentasaccharide **26**.

Introduction

Glycosaminoglycans (GAGs) are a family of highly sulfated, linear polyanionic molecules that are found on most animal cell surfaces as well as in the basement membranes and other extracellular matrixes. Heparin and heparan sulfate are the most widely studied members of this family. Heparin is exclusively synthesized by tissue mast cells and is stored in cytoplasmic granules, whereas the closely related molecule heparan sulfate is expressed on cell surfaces and throughout tissue matrices.[1] They are composed of repeating disaccharide units of 1 → 4 linked uronic acid and D-glucosamine (Figure 1). The uronic acid residues typically consist of 90% L-iduronic acid and 10% of D-glucuronic acid. The interaction of these polyanionic molecules with proteins plays an important role in several biological recognition processes, including blood coagulation, virus infection, cell growth, inflammation, wound healing, tumor metastasis, lipid metabolism, and diseases of the nervous system.[1,2]

The biosynthesis of heparin and heparan sulfate occurs by similar pathways.[2] Chain initiations occur in the Golgi apparatus. The first step in the pathway involves the attachment of a tetrasaccharide fragment to a serine residue in the core protein. This structure is then modified by a series of enzymatic transformations involving *N*-deacetylation followed by *N*-sulfation, substrate directed epimerization of glucuronic acid to iduronic acid moieties, and finally *O*-sulfation. Although these enzymatic modifications result in a mixture of very complex polysaccharides, structural studies have shown that heparin/heparan sulfates are composed of only 19 distinct disaccharide subunits, differing in their sulfation pattern and in the presence of either D-glucuronic or L-iduronic acid.

To date, more than one hundred heparin-binding proteins have been identified. With the exception of the antithrombin III-heparin interaction,[3] in which the minimal sequence of heparin pentasaccharide is required for binding, the structure and function of heparin interaction with proteins is poorly understood. This is mainly due to the complexity and heterogeneity of these polymers. With the discovery of increasing numbers of heparin-binding proteins, there is a need to characterize the molecular elements responsible for binding to a particular protein and modulating its biological activity. Since the first total synthesis of heparin pentasaccharide,[4] numerous synthetic methodologies have been reported for the synthesis of heparin fragments.[5] Most of the strategies involve traditional stepwise oligosaccharide synthesis in which protecting group and anomeric leaving group manipulations, intermediate workup, and

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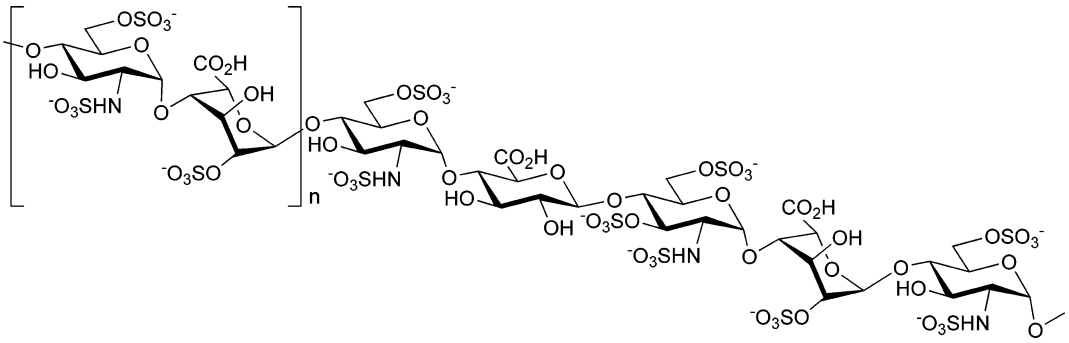
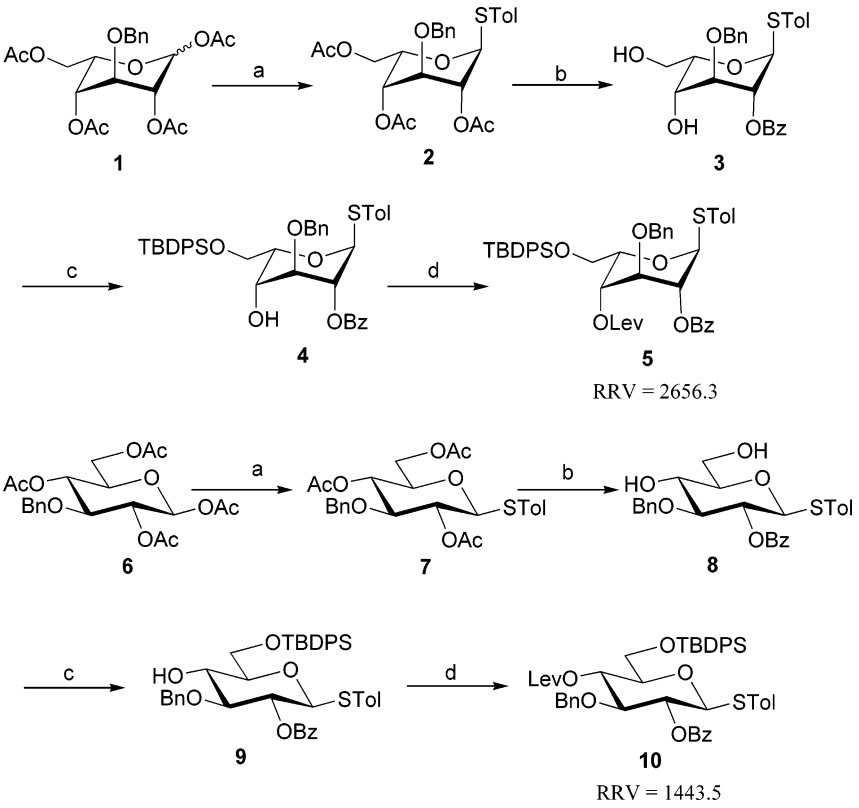


Figure 1. Schematic view of heparin.

Scheme 1. Preparation of the 1-Thio Uronic Acid Building Blocks **5** and **10**[a]



[a] Reagents and conditions: (a) TolSH, $BF_3{\cdot}Et_2O$, **2**: 93% (9:1 α/β), **7**: 87%; (b) i. NaOMe, MeOH; ii. $PhCH(OMe)_2$, *p*-TsOH, CH_3CN/DMF; iii. BzCl, Pyridine; iv. 60% TFA in H_2O, CH_2Cl_2, **3**: 78%, **8**: 81%; (c) TBDPSCl, Pyridine, **4**: 91%, **9**: 95%; d) Lev_2O, Pyridine, **5**: 89%, **10**: 91%.

purification in each step are required. Access to differentially substituted derivatives is important for dissecting recognition and activity, as recently demonstrated in the synthesis of heparin[6] and chondroitin sulfates.[7] A rapid and truly practical strategy capable of creating diverse derivatives of heparin oligosaccharides with differential sulfation pattern would be useful for detailed functional studies of these important molecules. Recently, we reported a reactivity-based one-pot method for complex oligosaccharides synthesis. In this methodology, the oligosaccharide is assembled rapidly by sequential addition of thioglycoside building blocks, with the most reactive one being added first.[8] The generality of thioglycosides makes them convenient and attractive building blocks due to their stability, accessibility, and compatibility.[9] Here we report an efficient one-pot strategy for the rapid assembly of representative heparin and heparan sulfate oligosaccharides.

Results and Discussion

For the synthesis of heparin and heparin sulfate, one has to overcome a range of synthetic difficulties imposed by the complex structure of heparin and heparin sulfate saccharides. Besides the careful design of protecting group strategy to enable the installation of sulfate groups on selected hydroxyl and amino

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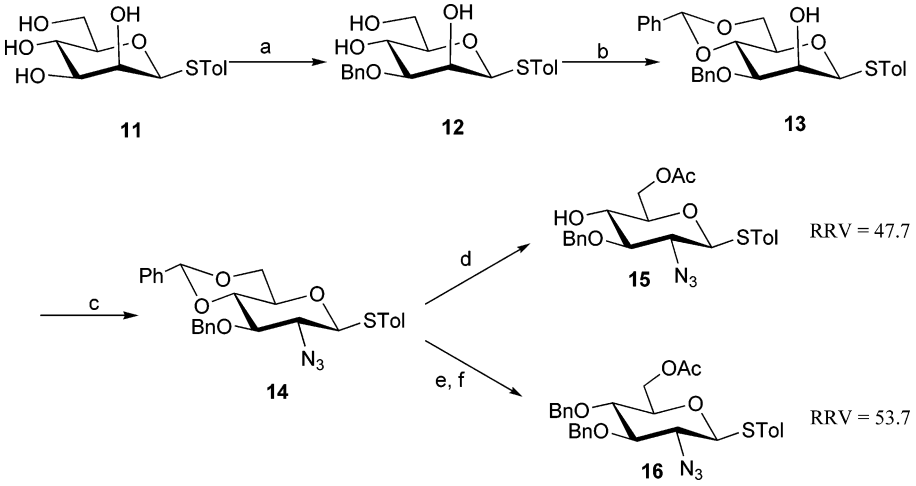
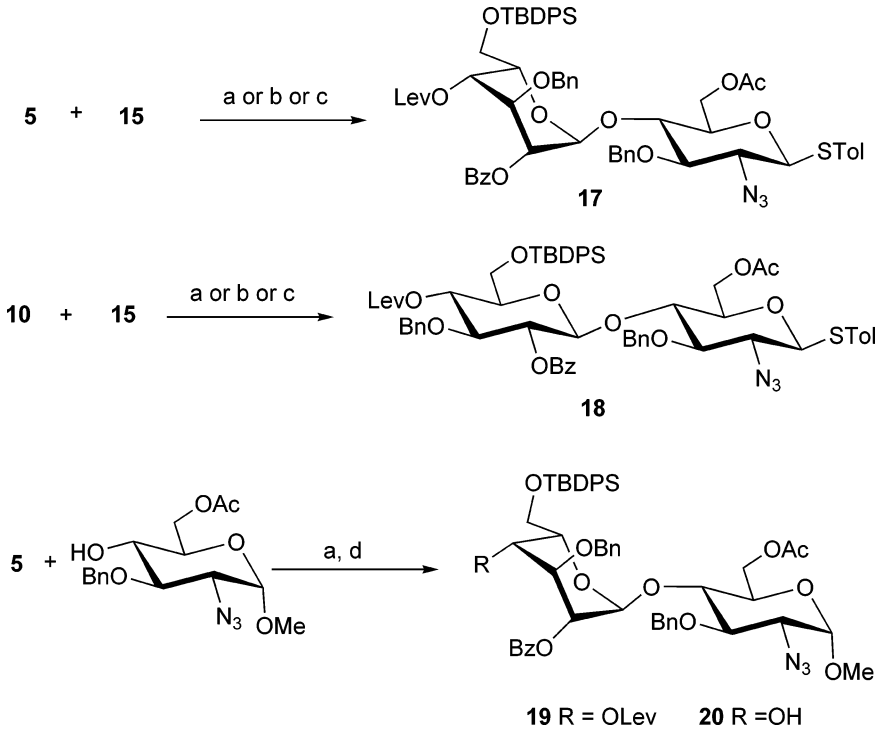
Scheme 2. Preparation of Azidoglucosyl Acceptors **15** and **16**[a]



[a] Reagents and conditions: (a) i. Bu_2SnO, Toluene ii. Bu_4NBr, BnBr, 65% in two steps; (b) $PhCH(OMe)_2$, CSA, 89%; (c) i. Tf_2O, Pyr-CH_2Cl_2, ii. NaN_3, DMF, 83% in two steps; (d) i. 80% AcOH, ii. AcCl, Pyridine, **15**: 89%; (e) $PhBCl_2$, Et_3SiH, CH_2Cl_2, 92%; (f) Ac_2O, Pyridine, **16**: 95%.

Scheme 3. One-Pot Synthesis of Disaccharide Derivatives **17**−**20**[a]



[a] Reagents and conditions: (a) NIS, TfOH, CH_2Cl_2, −45 °C to room temperature, **17**: 92%, **18**: 89%, **19**: 96%; (b) BSP, Tf_2O, CH_2Cl_2, −45 °C to room temperature, **17**: 72%, **18**: 75%; (c) *N*-(phenylythio)-ϵ-caprolactam, Tf_2O, CH_2Cl_2, −45 °C to room temperature, **17**: 85%, **18**: 88%; (d) NH_2NH_2/AcOH/Pyridine, **20**: 95%.

functions, the stereoselective construction of the glucosamine-uronic acid backbone has to be developed. The use of uronic acid building blocks as glycosyl donor[10] is limited and is often avoided,[11] because uronic acids are prone to epimerization, have the inherent low reactivity imposed by the C-5 carboxyl group, and complicate protecting group manipulations. Thus, in our synthetic approach, the formation of uronic acids by selective oxidation at the C-6 hydroxyl group was done after assembly of oligosaccharides. Control of the stereoselectivity of each glycosylation and generation of a significant reactivity difference between the thioglycosides are crucial for the successful synthesis of heparin oligosaccharides with the one-pot strategy. The reactivity of a sugar is highly dependent on its protecting groups and the anomeric activating group used. The reactivity differences between thioglycosides were mainly influenced by electron-donating and electron-withdrawing protecting groups. To this end, the hydroxyl groups to be sulfated were protected as acyl (acetyl and benzoyl) groups. Selective removal of acetate esters[12] in the presence of benzoates also enabled us to

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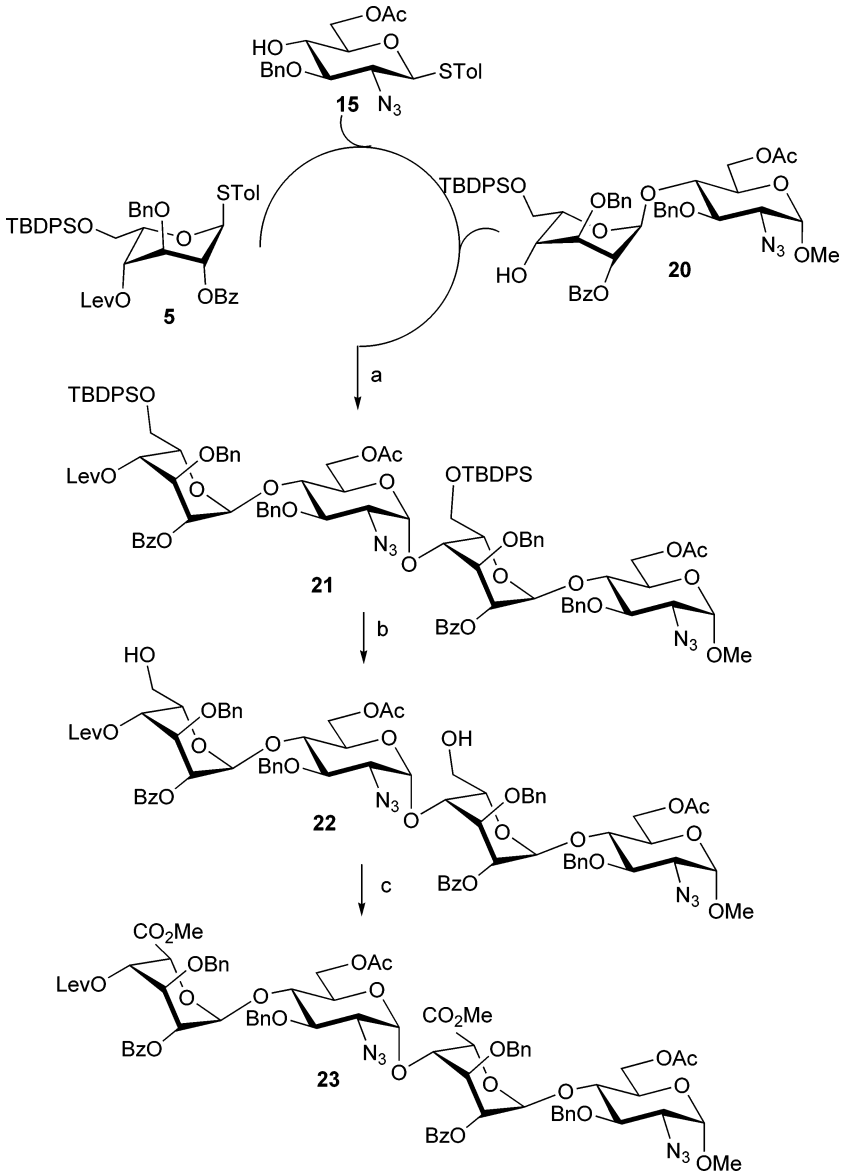
Scheme 4. One-Pot Synthesis of Tetrasaccharide Derivative **23**[a]



[a] Reagents and conditions: (a) NIS, TfOH, CH_2Cl_2, −45 °C to Room Temperature, 35%; (b) HF·Pyr, THF, 87%; (c) i. TEMPO, KBr, NaOCl, CH_2Cl_2, H_2O, ii. MeI, $KHCO_3$, DMF, 68% in two steps.

synthesize oligosaccharides with differential sulfation patterns. The primary hydroxyl groups to be selectively oxidized to uronic acids were protected as *tert*-butyldiphenylsilyl ethers. Finally, benzyl groups were installed on the remaining hydroxyls and the amino functionalities were masked as azides. Relative reactivity values (RRV) of monosaccharide building blocks were obtained by HPLC analysis with the established competitive assay method.[8a,13]

To test the above synthetic strategy, fully protected disaccharides **17**, **18**, tetrasaccharide **23**, and pentasaccharide **26** (a binding epitope for antithrobin III) were selected as model saccharides. Thus, L-idopyranosyl, D-glucopyranosyl, and azidoglucosyl thioglycosides were designed and prepared using the above synthetic strategy (Scheme 1). The known 1,2,4,6-*O*-tetra-*O*-acetyl-3-*O*-benzyl-α/*β*-D-idopyranoside[14] was used as starting material for the construction of **5**. The reaction of **1** with *p*-toluenethiol in the presence of BF_3·Et_2O gave **2** in 93% yield. Standard removal of acetate esters in **2** and formation of the 4,6-*O*-benzylidene acetal, followed by protection of the C-2 hydroxyl as a benzoate ester, which was chosen for its participating group assistance in the forthcoming glycosylation reaction. Subsequent acidolysis of the cyclic acetal afforded idopyranosyl thioglycoside **3** in 78% yield. Using standard methods, introduction of the *tert*-butyldiphenylsilyl group at C-6 (91%) and introduction of the levulinyl group at C-4 provided fully protected idopyranosyl thioglycoside **5** (RRV = 2656.4) in 89% yield. This route was also applied to the synthesis of glucopyranosyl thioglycoside **10** (RRV = 1443.5) starting from the known 1,2,4,6-*O*-tetra-*O*-acetyl-3-*O*-benzyl-*β*-D-glucopyranoside[15] (Scheme 1).

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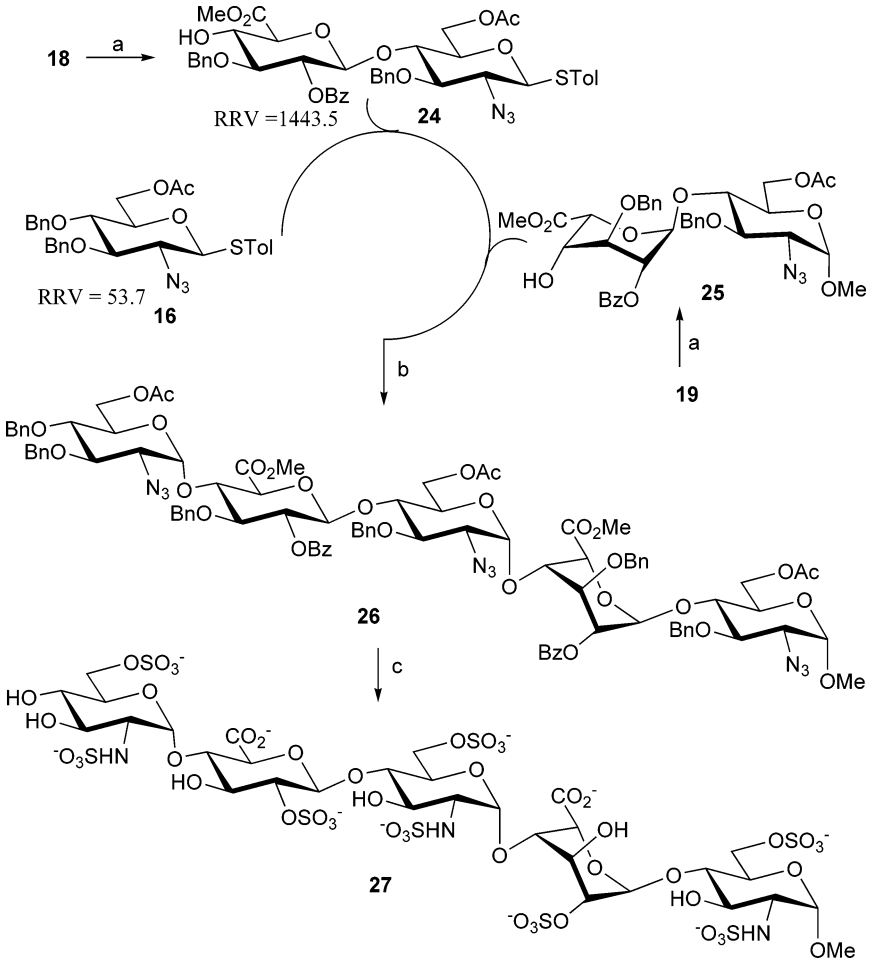
Scheme 5. One-Pot Synthesis of Pentasaccharide Derivative **27**[a]



[a] Reagents and conditions: (a) i. HF·Pyr, THF; ii. TEMPO, KBr, NaOCl, CH_2Cl_2, H_2O, then MeI, $KHCO_3$, DMF; iii. NH_2NH_2/AcOH/Pyridine, **24**: 45%, **25**: 77%; (b) i. NIS, TfOH, CH_2Cl_2, −45 °C to room temperature; ii. NIS, TfOH, CH_2Cl_2, −45 °C to room temperature, 20%; c) i. LiOOH, THF; ii. $Et_3N{\cdot}SO_3$, DMF; ii. H_2, Pd/C; *iv*- Pyr·SO_3, H_2O, 33%.

Next azidoglucosyl thioglycoside building blocks **15** and **16** were synthesized following our previously reported methods (Scheme 2).[5f] Briefly, the C-3 hydroxyl was selectively protected as a benzyl ether after di-*n*-butyltin oxide activation.[16] Direct benzylidination of resulting **12** afforded **13** in 89% yield. Azidoglucosyl thioglycoside derivative **14** was formed through a two-step sequence: triflation of the free hydroxyl was followed by nucleophilic substitution with NaN_3 in DMF gave compound **14** in 83%. Removal of 4,6-*O*-benzylidene acetal and regioselective introduction of the acetyl group at C-6 afforded the fully protected azidoglucosyl acceptor **15** (RRV = 47.7). In a similar manner, selective opening of 4,6-*O*-benzylidene acetal in **14** using $PhBCl_2$ and Et_3SiH, followed by acetylation at C-6 afforded azidoglucosyl donor **16** (RRV = 53.7).

With all monosaccharide building blocks in hand, we turned our attention to the one-pot synthesis of heparin oligosaccharides. First, disaccharide formation by different thioglycoside activators was examined, using *N*-iodosuccinimide/trifluoromethanesulfonic (triflic) acid (NIS/TfOH),[8a] benzenesulfinyl piperidine (BSP)/triflic anhydride (Tf_2O)[8b] or *N*-(phenylythio)-ϵ-caprolactam/Tf_2O.[8c] These activators were previously used in several reactivity-based one-pot syntheses.[8] Because the reactivity of idopyranosyl thioglycoside **5** (RRV=2656.3) or glucopyranosyl thioglycoside **10** (RRV=1443.5) is much higher than that of **15** (RRV = 47.7), glycosylation of donor **5** or **10** with azidoglucosyl thioglycoside acceptor **15** afforded the desired disaccharide **17** or **18** in excellent yield and stereoselectivity with no self-coupling of **5** (Scheme 3). Slightly better yield was observed in both cases using NIS/TfOH as the activator. In addition, disaccharide acceptor **20** was synthesized in a straightforward manner (Scheme 3). Glycosylation of **5** with methyl 2-azido-3-*O*-benzyl-2-deoxy-α-D-glucopyronoside[10a] in the presence of NIS/TfOH afforded desired disaccharide **19** in 96% yield. Removal of the levulinyl group with NH_2NH_2/AcOH/Pyridine afforded disaccharide acceptor **20** in 95% yield.

In an effort to extend the application of one-pot synthesis, syntheses of tetrasaccharide and pentasaccharide were next examined. For the one-pot tetrasaccharide synthesis (Scheme 4), fully protected idopyranosyl donor **5** was first coupled with azidoglucosyl acceptor **15** in the presence of NIS/TfOH at −45 °C followed by slow warming to room temperature. After 3 h, α-methyl disaccharide acceptor **20** was added, followed by the addition NIS/TfOH at the same temperature. The fully protected tetrasaccharide **21** was obtained in 35% yield. With this methodology, protecting group and anomeric leaving group manipulations, intermediate workup, and purification can be

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avoided. Removal of the silyl ether protection group with HF-pyridine afforded **22** in 87% yield. The resulting primary hydroxyl groups were oxidized with 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) and NaOCl as a co-oxidant[5g] under basic conditions (pH = 10). The corresponding carboxyl groups were then esterified in the presence of MeI and $KHCO_3$ to give the desired fully protected tetrasaccharide **23** in 68% yield over two steps.

When the same approach was applied to the one-pot synthesis of the pentasaccharide, unfortunately, the yield in the first glycosylation was very low. This was partially attributed to the bulky silyl group at C-6 on **18**, which partially blocks the C-4 hydroxyl. Changing the silyl protection at C-6 to a smaller group was expected to increase the yield. Thus, the TBDPS groups of compounds **18** and **19** were deprotected using HF·pyridine in THF and oxidation of the resulting hydroxyl groups with TEMPO, NaOCl followed by methylation, and finally removal of levulinate afforded disaccharides **24** and **25** in 45 and 77% yield, respectively. For the one-pot pentasaccharide synthesis (Scheme 5), azidoglucosyl donor **16** (RRV = 53.7) was first coupled with disaccharide acceptor **24** (RRV = 18.2), and then α-methyl disaccharide acceptor **25** was added to the reaction mixture. Under these conditions, the fully protected pentasaccharide **26** was obtained in 20% yield. The corresponding *O*-sulfates were obtained by consecutive saponification with LiOOH and *O*-sulfation with triethylamine-sulfur trioxide followed by palladium catalyzed hydrogenolysis and *N*-sulfation with pyridine-sulfur trioxide to provide the desired heparin pentasaccharide **27**. It is noted, however, that selective deprotection of the acetyl group, the benzoyl group, the methyl ester group, and the benzyl group can be carried out with known procedures to create different sulfation patterns.

Conclusion

In conclusion, we have developed a one-pot strategy for the synthesis of heparin-like oligosaccharides. Carefully designed monosaccharide building blocks (e.g., **5**, **10**, **15**, and **16**) with well-defined reactivity were successfully used in the representative one-pot synthesis of disaccharides **17**, **18**, tetrasaccharide **23**, and pentasaccharide **26**. No self-coupling of the building blocks was observed in each case, illustrating the importance of quantitative reactivity determination for the implementation of programmable one-pot synthesis. Heparin pentasaccharide derivative **27** was obtained after global deprotection and sulfation. We believe that this new strategy has potential for rapid synthesis of various heparin analogs for the study of their biological properties.

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Supporting Information Available: Full experimental and characterization details for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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babA alleles reflects long-term adaptation to the types of receptors available in local populations, punctuated by fine-tuning of adherence during persistent infection of any individual host. Although adherence should benefit *H. pylori* by allowing better access to nutrients and delivery of effector molecules, tight adherence may be deleterious when host responses are robust. Recently, *H. pylori* infection and gastritis were found to promote gastric mucosal expression of inflammation-associated sialyl-Lewis x/a antigens (*3*), in competition with the fucosylated antigens that were studied here (*10*). Changes in BabA adhesins that help strains adapt to host gastric environments could arise by point mutation or short patch recombination between strains, or between divergent alleles in the same strain as illustrated by the ALeb binding transformant P445G [(*11*–*13*) and this study]. Such new *babA* alleles will often differ from their parents in affinity, and those that are best suited to the local gastric environment (whether they are of higher or lower affinity) will be selected. Such flexibility should help to ensure rapid adaptation of *H. pylori* populations to the glyco-phenotype and host response of each infected person.

The postulated cycles of selection for decreased and then increased adherence during chronic infection and transmission from one person to another should result in retention of the ALeb, BLeb, and Leb generalist binding modes in most human populations because of the abundance of A, B, and O blood groups in them. In explaining the abundance of Leb-only specialist strains in South America, we note that Amerindians of this region are unique in being almost entirely of blood group O phenotype (*14*). One might invoke the idea of selection for dedicated specialists in this population. However, because the distribution of Leb affinities of Peruvian specialists is similar to those of generalists everywhere, we prefer an alternative explanation, which invokes recurrent cycles of selection for loss and restoration of binding activity: Only restoration of Leb binding activity would be selected for in any uniformly blood group O population; thus generalist binding would be lost by attrition.

Most alleles of housekeeping genes in the Peruvian *H. pylori* strains studied here were closely related to those found in Spanish strains but not those of Asian strains (*15*, *16*). This implies descent of these Peruvian strains mainly from European strains (*16*). If this is correct, most Peruvian specialist *babA* alleles may have arisen by mutation and/or recombination over only the last ~500 years. We propose that such rapid evolvability of the BabA adhesin in response to host mucosal glycosylation patterns fine-tunes *H. pylori* strains to their individual hosts, helps them to avoid the most deleterious of host responses, and contributes importantly to the extraordinary chronicity of human *H. pylori* infection worldwide.

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17. We thank P. Falk for valuable discussions, F. Lindh, IsoSepAB (Tullinge, Sweden), and T. Norberg for synthesis of the A7 conjugate, A. Parkinson for Alaska Natives strains, P.-G. Nyholm for structural predictions of blood group antigens, I. Sjöström for technical assistance, and A. Kidd for proofreading. This work was supported by the Mizutani Foundation Glycoscience Award 2003, the Commission of the European Union (QLK2-2001-01216-ADRI), Umeå University Biotechnology Fund, the County Council of Västerbotten and Stiftelserna JC Kempeoch Seth M Kempes Minne (T.B.), the Swedish Medical Research Council [11218 (T.B.), 12628 (S.T.), and 7902 (I.C.)], the Swedish Cancer Society [4101-B00-03XAB (T.B.) and 4128-B99-02XAB (S.T.)], Swedish Foundation for Strategic Research (SSF) programs [Glycoconjugates in Biological Systems (N.R., T.B., and S.T.) and Infection and Vaccinology (M.A.-H. and T.B.)], the Swedish Medical Society (S.T.), the Swedish Society for Medical Research (S.V. and M.A-H.), the J. C. Kempe Memorial Foundation, the Royal Swedish Academy of Sciences (M.A.-H.), and NIH [grants RO1 AI38166, RO3 AI49161, RO1 DK53727, RO1 DK63041 (D.B.), P30 DK52574 (Washington University), and ROIGM62370 (M.B.)]. The 66 *babA* sequences in Fig. 2 have been deposited in GenBank under accession AY648168-AY648233.

Supporting Online Material

www.sciencemag.org/cgi/content/full/305/5683/519/DC1
Materials and Methods
Figs. S1 to S12
Tables S1 to S8

6 April 2004; accepted 23 June 2004

A Synthetic Conjugate Polysaccharide Vaccine Against *Haemophilus influenzae* Type b

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Glycoconjugate vaccines provide effective prophylaxis against bacterial infections. To date, however, no commercial vaccine has been available in which the key carbohydrate antigens are produced synthetically. We describe the large-scale synthesis, pharmaceutical development, and clinical evaluation of a conjugate vaccine composed of a synthetic capsular polysaccharide antigen of *Haemophilus influenzae* type b (Hib). The vaccine was evaluated in clinical trials in Cuba and showed long-term protective antibody titers that compared favorably to licensed products prepared with the Hib polysaccharide extracted from bacteria. This demonstrates that access to synthetic complex carbohydrate–based vaccines is feasible and provides a basis for further development of similar approaches for other human pathogens.

Haemophilus influenzae type b (Hib) is an important human pathogen and was prevalent in developed countries until the introduction of successful conjugate vaccines during the 1990s (*1*). However, in developing countries more than 600,000 infant deaths occur annually as a result of Hib-induced pneumonia or meningitis (*2*). Extensive use of the polysaccharides as vaccines has offered a useful way to protect adults and older children (*3*–*5*), and further improvement in generating long-lasting immunity, especially in infants, has been achieved by covalently coupling the polysaccharide to carrier proteins (*6*). In fact, the high level of success attained by Hib glycoconjugate vaccine (*7*) has been quickly followed by similar approaches to meningococcal group C (*8*) and *Streptococcus pneumoniae* (*9*). Many candidate vac-

cines against other pathogens using the same principles are currently at different stages of research (*10*, *11*).

The fragment of the Hib capsular polysaccharide used in some of the licensed vaccines can be as short as five ribosylribitol-phosphate repeating units (*12*). The ability of synthetic carbohydrate chemistry to mimic such fragments has been demonstrated in several laboratories with the use of stepwise multistep preparation (*13*–*15*); the resulting synthetic antigens have served as components of candidate vaccines that have proven efficient in generating immunity in animals (*16*, *17*). We set out to develop a synthetic methodology amenable to large-scale good manufacturing practice (GMP) production of antigens by reassembling Hib polysaccharide fragments. The previous process was redesigned to include a synthetic pathway with a reduced number of reaction and chromatography purification steps. We also identified a potentially superior method for oligomerization of the ribosylribitol-phosphate repeating unit, in which the saccharide fragment encompassing the key conformational epitope can be obtained in a single step.

To this end, we selected suitably protected ribitol derivative **1** and ribose acetate **2** because they best fit the criteria for large scale production as synthetic intermediates and could be more readily purified than others derived from D-glucose (Fig. 1). The use of crystalline peracetylated β-D-ribofuranose, **2**, was found to readily glycosidate ribitol derivative **1** to provide **3a** (*18*). The successful large-scale synthesis of ribosylribitol derivatives **4** and **5** from **3a** as described in Fig. 1 thus represented one of the key features of our strategy.

Although construction of oligomers with controlled numbers of repeating units by solution and solid-phase techniques was possible in small quantities, their large-scale syntheses proved more difficult. To overcome this, we undertook a one-step polycondensation reaction with the use of H-phosphonate chemistry (*19*). Thus, the phosphate-containing end residue **4** and H-phosphonate derivative **5** were oligomerized in high yield and purity with the use of pivaloyl chloride as a polycondensation reagent (Fig. 1). Although this reaction is complex, it could be managed to generate the desired oligomers, avoiding several competing side reactions, such as O-acylation.

Synthetic oligomers of **6a** with an average of eight repeating units were reproducibly obtained in high yield (80%) after purification by size exclusion chromatography. Deprotection and azide reduction of **6a** to the amine **6b**, which, after treatment with 3-maleimidopropionic acid N-hydroxysuccinimide ester, gave **7** (Fig. 2). The overall process accomplished under GMP proceeded with a high yield and was amenable to a 100-g scale per batch.

A vaccine prototype was subsequently produced by conjugating synthetic antigen **7** with thiolated human serum albumin (HSA) (*20*). The polyribosylribitol phosphate (sPRP)–HSA conjugate was used for coating enzyme-linked immunosorbent assay (ELISA) plates to screen for anti-Hib activity (*21*) of sera obtained from rabbits immunized with commercially available vaccines [Vaxem-Hib (Chiron, Emeryville, CA) and Hiberix (Glaxo Smith Kline Biologicals, Rixensart, Belgium)] and human antibodies against Hib obtained from immunized children (Vaxem-Hib). All sera showed equivalent recognition of sPRP-HSA and natural PRP–HSA conjugates, demonstrating that the synthetic oligosaccharide possessed the relevant antigenic epitopes for antibody binding recognition (*22*).

As a potentially more relevant protein carrier for synthetic oligosaccharides, a tetanus toxoid (TT) conjugate **9** was evaluated in animals. The glycoconjugate was obtained through the thiolation of TT lysine ε-amino groups as for HSA, and this sPRP-TT conjugate was immunogenic in rabbits with a wide range of sPRP/protein ratios, inducing a strong and specific antibody response (*22*).

At this point, we identified four key issues that allowed us to accomplish further development of sPRP-TT as a vaccine candidate: (i) a synthetic pathway to disaccharide derivative **5** with only one chromatographic purification step, making it amenable to large-scale GMP production, (ii) a single-step, high-yielding polycondensation reaction between **4** and **5** for the elongation of the oligosaccharide chain, (iii) a method for careful removal of protective groups, yielding highly pure sPRP **7**, and lastly (iv) a conjugation process to TT carrier that incorporated sPRP in good yields.

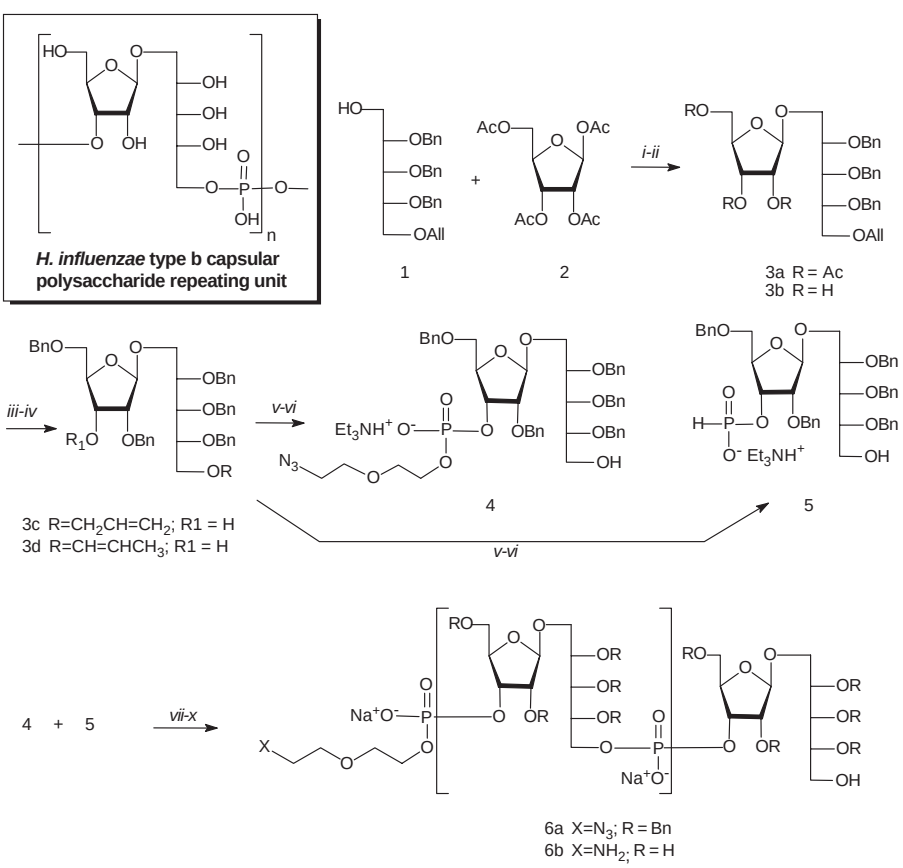

Fig. 1. Synthetic pathway leading to oligomeric polyribosylribitol phosphate **6**. Reagents and conditions (Et, ethyl; Bn, benzyl; Bu, butyl; Ac, acetyl; and Piv, pivaloyl): (i) BF_3Et_2O and CH_2Cl_2; (ii) CH_3ONa and CH_3OH; (iii) BnCl, Bu_2SnO, NaH, and Bu_4NI; (iv) tBuOK and dimethyl sulfoxide (DMSO) at 100°C; (v) PCl_3, imidazole, CH_3CN for **5** and $N_3(CH_2)_2O(CH_2)_2OH$ for **4**, followed by I_2 oxidation; (vi) AcOH-H_2O at 80°C; (vii) PivCl and pyridine; (viii) I_2, pyridine, H_2O then gel filtration on LH-20; (ix) H_2, Pd-C, and EtOH-H_2O-EtOAc-AcOH at 1.5 atm; (x) cation exchange chromatography on Sephadex SP-C25.

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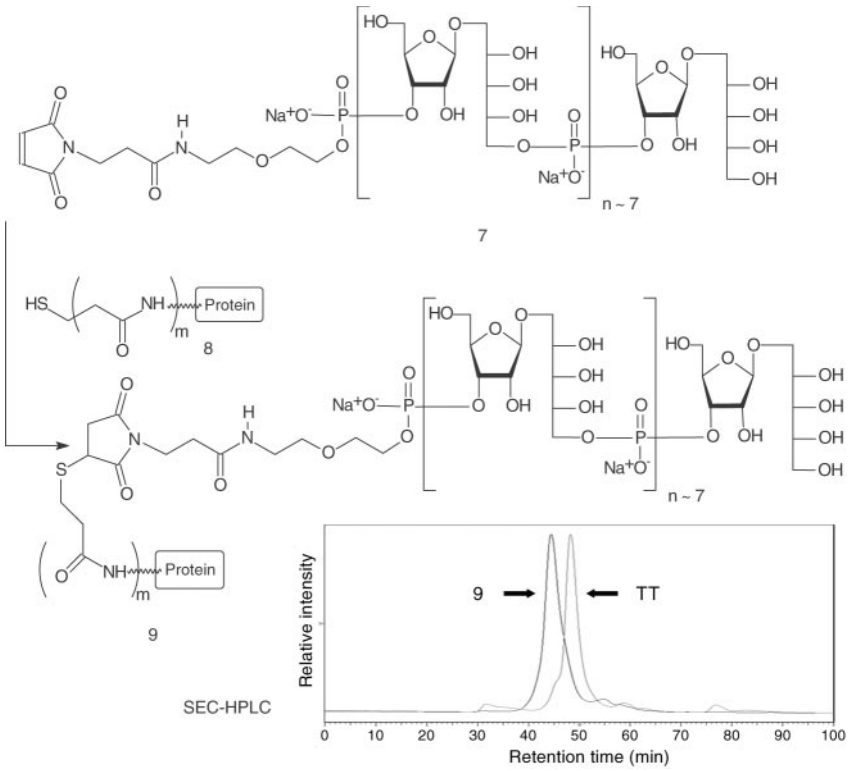

Fig. 2. Conjugation of the maleimido-functionalized polyribosylribitol phosphate **7** obtained from **6b** after coupling with 3-maleimidopropionic acid N-hydroxysuccinimide (DMSO, >95% conversion). 1,4-Conjugate addition of thiolated protein **8** onto **7** provided conjugate **9**. The shift in the molecular weight for TT could be observed in size exclusion chromatography–high performance liquid chromatography (TSK-5000-1 column) for conjugate **9** (PRP/TT ratio of 1/2.6).

The sPRP-TT conjugate vaccine with 10 μg of sPRP and a sPRP-to-TT ratio of 1/2.6 by weight was next used as part of a phase I clinical trial in 40 adult volunteers after rigorous toxicological assessment in animals (*22*). A second phase I trial in adults with the vaccine plus aluminum phosphate was also performed. Both formulations of sPRP-TT were shown to be safe in adults. The average immunoglobulin G (IgG) titers obtained from our synthetic antigens (*23*) displayed a behavior similar to that of the control vaccine (Vaxem-Hib); the results obtained from this initial evaluation (table S1) prompted the initiation of a full clinical evaluation. All trials were performed as recommended and according to good clinical practice (*24*). Generally, they were randomized and double-blind and used a control vaccine and at least two different batches of the test vaccine (*25*).

The next phase I clinical trial consisted of a single vaccine dose in 133 4- to 5-year-old children previously unvaccinated against Hib. This was followed by a phase II trial using 1041 children. A substantial postvaccination increase in the anti-Hib IgG titers was observed in both trials, and a significant increase in the bactericidal activities of the sera (*26*) was reached after the administration of a single vaccine dose in three groups of children. The similarities of these with previous studies (*27*) again indicated that the sPRP-TT

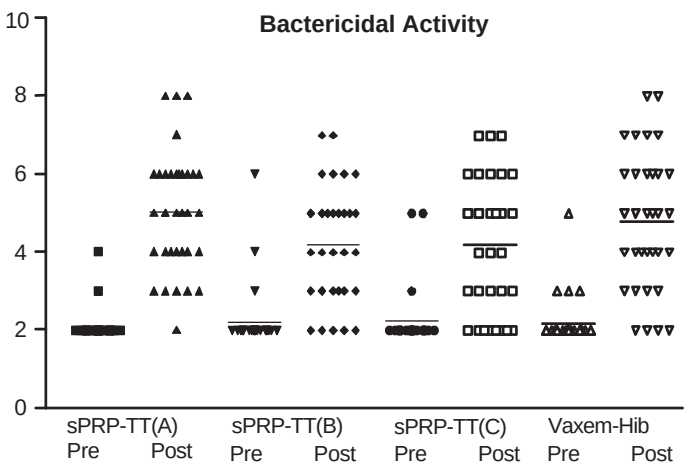

Fig. 3. Bactericidal activity of immune serum against Hib (Eagan strain) obtained during a phase I trial conducted with children 4 to 5 years old in the province of Camaguey, Cuba, in the winter of 2002. The trial was conducted with 133 preschool children volunteers with the informed written consent of both parents. The trial was performed on a double-blind basis and all subjects were divided in four groups. Three groups (A to C) received a single dose of s-PRP-TT without any adjuvant (lots EH1024E, EH1026E, and EH1025E, respectively). The control vaccine (Vaxem-Hib) was administered to another group. The serum was obtained before and 4 weeks after the immunization. The plots are the reciprocal of the dilution killing 50% of the bacteria according to (*26*, *27*).

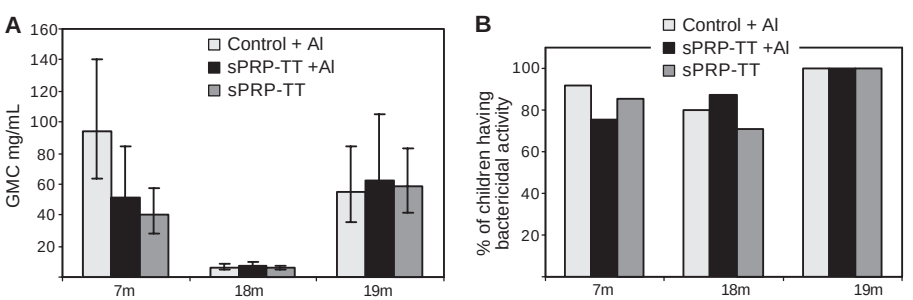

Fig. 4. Geometric means concentration (GMC) of serum IgG antibodies against Hib (**A**) and % of children having bactericidal anti-Hib in their serum (**B**) after the primary series of immunization (7 months), a year after the preimmunization (18 months), and after a booster dose (19 months). A phase I clinical trial was performed with 139 infants in the Camaguey province east of Havana. Infants were enrolled in five groups receiving three doses at 2, 4, and 6 months of age. One group received control vaccine (Vaxem-Hib). Two groups received s-PRP-TT plus aluminum phosphate, and two other groups received s-PRP-TT alone. Blood was drawn at 7, 18, and 19 months for the evaluation of the immune status. At 18 months, all infants received a single booster dose of sPRP-TT irrespective of group. Bars indicate the 95% confidence interval.

vaccine was both as safe and as immunogenic as the commercial control vaccine (Fig. 3).

The above results set the stage for more detailed clinical assessment of the vaccine in a targeted infant population. Thus, a phase I trial was initiated with 139 2-month-old infants who received three vaccine doses scheduled at 2, 4, and 6 months, as recommended for other conjugate anti-Hib vaccines. The test vaccine induced a strong and bactericidal antibody response against Hib in infants (Fig. 4) that fell to values ranging from 5 to 7 μg/mL at 18 months of age but remained at least five times that required for long-term protection (Fig. 4A). A booster dose with sPRP-TT applied to all groups increased the antibody against Hib titers by 10-fold. Thus, the capacity of sPRP-TT to prime an effective immune response against Hib was demonstrated.

In a second phase II trial, a total of 1141 infants distributed in three groups received three doses of either sPRP-TT conjugate, sPRP-TT mixed with aluminum phosphate, or the control vaccine (Vaxem-Hib). Of the test infants, 99.7% reached antibody titers above 1 μg/mL, which is considered appropriate for long-lived protection against Hib (*28*, *29*). The mean IgG anti-PRP titer was 27.4 μg/mL for all infants vaccinated with the sPRP-TT, which is consistent with previously reported clinical trials (between 7.67 and 35 μg/mL) for anti-Hib vaccines without adjuvant (*30*, *31*).

The present study demonstrates that a synthetic capsular polysaccharide antigen can be produced on a large scale under GMP conditions and used to manufacture an effective vaccine for human use. The resulting conjugate vaccine incorporating a synthetic bacterial carbohydrate antigen was demonstrated to be as safe and immunogenic in humans as already-licensed vaccines incorporating the native polysaccharide (*32*–*34*). Access to synthetic complex carbohydrate-based vaccines is therefore feasible and provides an alternative strategy in the fight against Hib infections. It also sets the stage for further development of similar approaches against other human pathogens.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/305/5683/522/DC1
Materials and Methods
Fig. S1
Table S1

31 December 2003; accepted 23 June 2004

Large-Scale Copy Number Polymorphism in the Human Genome

Jonathan Sebat,[1] B. Lakshmi,[1] Jennifer Troge,[1] Joan Alexander,[1] Janet Young,[2] Pär Lundin,[3] Susanne Månér,[3] Hillary Massa,[2] Megan Walker,[2] Maoyen Chi,[1] Nicholas Navin,[1] Robert Lucito,[1] John Healy,[1] James Hicks,[1] Kenny Ye,[4] Andrew Reiner,[1] T. Conrad Gilliam,[5] Barbara Trask,[2] Nick Patterson,[6] Anders Zetterberg,[3] Michael Wigler[1]*

The extent to which large duplications and deletions contribute to human genetic variation and diversity is unknown. Here, we show that large-scale copy number polymorphisms (CNPs) (about 100 kilobases and greater) contribute substantially to genomic variation between normal humans. Representational oligonucleotide microarray analysis of 20 individuals revealed a total of 221 copy number differences representing 76 unique CNPs. On average, individuals differed by 11 CNPs, and the average length of a CNP interval was 465 kilobases. We observed copy number variation of 70 different genes within CNP intervals, including genes involved in neurological function, regulation of cell growth, regulation of metabolism, and several genes known to be associated with disease.

Many of the genetic differences between humans and other primates are a result of large duplications and deletions (*1*–*3*). From these observations, it is reasonable to expect that differences in gene copy number could be a significant source of genetic variation between humans. A few examples of large duplication polymorphisms have been reported (*4*). However, because of previous limitations in the power to determine DNA copy number at high resolution throughout the genome, the extent to which copy number polymorphisms (CNPs) contribute to human genetic diversity is unknown.

In our previous studies of human cancer with the use of representational oligonucleotide microarray analysis (ROMA), we have detected many genomic amplifications and deletions in tumor genomes when analyzed in comparison to an unrelated normal genome (*5*), but some of these genetic differences could be due to germline CNPs. To correctly interpret genomic data relating to cancer and other diseases, we must distinguish abnormal genetic lesions from normal CNPs.

We used ROMA to investigate the extent of copy number variation between normal

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TETRAHEDRON LETTERS

The First Synthesis of Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-Ser — a Newly Discovered Component of α-dystroglycan

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Abstract: Glycopeptide (**1**), Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-Ser, was synthesized using a chemoenzymatic strategy. Galβ1-4GlcNAcβ1-2Man trisaccharide was prepared using glycosidase assisted oligosaccharide synthesis. After coupling of this trisaccharide with a serine derivative by chemical glycosylation, sialic acid was introduced using sialyltransferase to produce a tetrasaccharide serine derivative. Removal of protecting group afforded glycopeptide (**1**). Use of a chemoenzymatic strategy allowed for the elimination of numerous synthetic steps and efficient preparation of the target compound.

Neu5Acα2-3Galβ1-4GlcNAcβ1-2Man-Ser/Thr which binds to a laminin molecule in the extracellular matrix was discovered between the 317th - 488th amino acid residues of α-dystroglycan[1] by Endo *et. al.*.[2] *O*-linked sugar chains which are bound via a Man-Ser/Thr linkage occur frequently in yeast but are extremely rare in mammals. The carbohydrate moieties of α-dystroglycan are thought to be essential to its biological functions.[3] For the purpose of elucidating the biological role of Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα-Ser (**1**), we have developed a short and direct synthesis of **1**.

The synthetic plan for the tetrasaccharide derivative bound to a serine residue (**1**) is shown in Figure 1. Trisaccharide (Galβ1-4GlcNAcβ1-2Man) was obtained by glycosidase assisted synthesis and was then converted into glycosyl donor **3**. Coupling of **3** and serine derivative **4** was performed by chemical glycosylation. Finally, the sialic acid residue was introduced using sialyl transferase.

The synthesis of thioglycoside **3** by use of glycosidase was performed as follows: disaccharide **5** was prepared according to our previous report.[4] Reverse hydrolysis of mannose and *N*-acetylglucosamine in the presence of *β-N*-acetylglucosaminidase from *Bacillus circulans* gave GlcNAcβ1-2Man (**5**) and GlcNAcβ1-6Man in 0.5% and 2.4% yield, respectively. Thiophenyl group was introduced to the anomeric position of the mannose residue in **5** to give **6**[6] in 2 steps in 92 % overall yield. Subsequent removal of the acetyl groups using NaOMe/MeOH at room temperature gave **7**[5] in quantitative yield. Galactosylation of **7** was performed by

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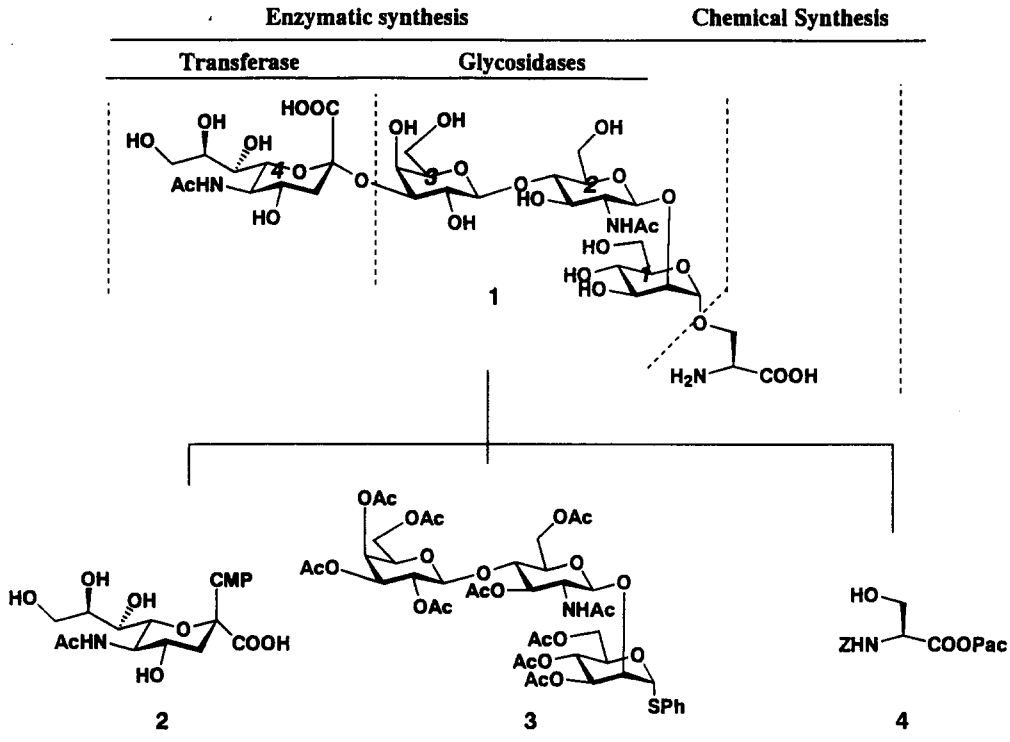
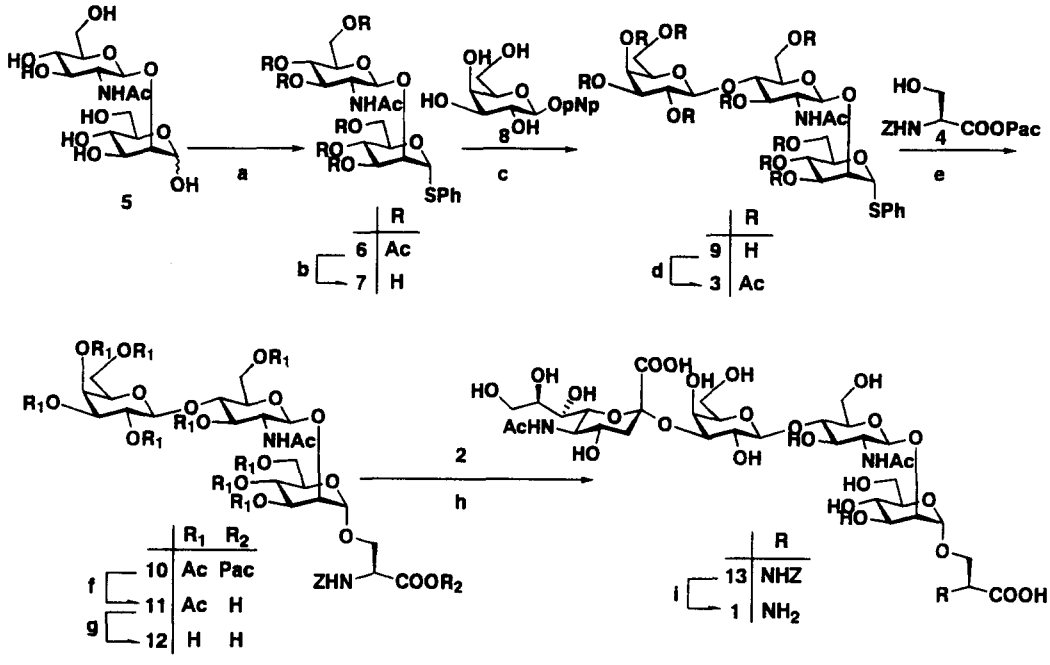


Figure 1

transglycosylation using β-galactosidase. When the disaccharide **7** (100 mg, 0.21 mmol) and *p*-nitrophenyl (pNp)-galactoside **8** (64 mg, 0.21 mmol) in 0.1 M phosphate buffer (pH 6.0) containing 10 % DMSO were incubated in the presence of β-galactosidase from *bifidobacterium bifidum* (12 U), galactose residue was introduced to the C-4 position of *N*-acetylglucosamine in the disaccharide **7** regioselectively to give **9**[5] (20 mg, 0.03 mmol) in 15 % yield (47 % of **7** was recovered). Treatment of **9** with acetic anhydride and pyridine gave the trisaccharide **3**[5] in 83 % yield. Coupling of **3** and the serine derivative **4** whose α-carboxyl group is protected with a Phenacyl (Pac) group was performed by using 5eq. of NIS and 1eq. of TfOH[6] in CH_2Cl_2 at -78 °C to give the glycopeptide **10**[5] in 98 % yield. The α-configuration of the newly formed glycosidic bond was confirmed by the $^1J_{C\text{-}H}$ and $^3J_{H1\text{-}H2}$ coupling constants of the NMR spectrum (172 Hz, and ~0 Hz, respectively). Removal of all protecting groups except the Z group were performed as follows: the Pac group of the serine residue in **10** was removed by using Zn/AcOH[7] at room temperature, and deacetylation of **11** was performed by using NaOMe/MeOH at 0 °C to give compound **12**[5] (overall yield 53 %). Epimerization of the serine residue of **12** under these conditions was not observed by HPLC or ^{1}H NMR spectroscopy. Although the enzymatic sialylation might be performed by either sialidase[8] or sialyltransferase[9], we chose the latter since high reaction yields and regioselectivities were expected.[9] Sialylation of **12** was performed as follows: a mixture of **12** (11 mg, 14.3 μmol), CMP-NeuAc **2** (22 mg, 35.8 μmol), and α-2,3-sialyltransferase[10] (300 mU) in 0.05 M cacodylate buffer (pH 6, 2 mL) containing 0.05 M NaCl and bovine serum albumin (1.9 mg) was incubated at 37 °C for 2 days. The reaction was monitored by an HPLC fitted with an ODS column and a UV monitor. The mixture was

purified by an HPLC fitted with an ODS column (20 % aq. CH_3CN containing 0.1% TFA) to give compound **13**[4] (13 mg, 12.3 μmol) in 86 % yield. The structure of the sialylated compound **13** was determined by ^1H and ^{13}C NMR spectroscopy to be on α-2,3-linkage (sialic acid residue of H-3^e: 2.69 ppm, H-3^a: 1.76 ppm, and C-3: 41.03 ppm). Removal of the Z group was accomplished by hydrgenolysis using H_2 / Pd-black in water to give target compound **1**[4] in quantitative yield.



Scheme 1: conditions; a) 1) Ac_2O, Pyridine. 2) PhSH, $SnCl_4$, CH_2Cl_2. b) NaOMe, MeOH. c) β-galactosidase from *b. bifidum*, aq. DMSO. d) Ac_2O, Pyridine. e) NIS, TfOH, CH_2Cl_2. f) Zn, aq. AcOH. g) NaOMe, MeOH. h) α-2,3-(N)-sialyltransferase from rat, recombinant, cacodylate buffer pH 6.i) Pd-black, H_2, H_2O.

In summary, Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα-Ser (**1**) was synthesized in only 9 steps from the disaccharide **5** using a chemoenzymatic strategy. Use of both a glycosidase and a transferase in the synthesis of the oligosaccharide portion ensured formation of glycosidic bonds in high stereo- and regio-selectivity and eliminated many complicated synthetic steps as necessary in traditional chemical oligosaccharide synthesis. Moreover, by use of the aromatic protecting groups, the products were made easy to separate by HPLC using an ODS column due to the resulting hydrophobicity and their ability to be monitored using a UV detector.

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5 ^{1}H and ^{13}C NMR data for key compounds are described below. NMR spectra were measured on a UNITY INOVA 500 spectrometer in $CDCl_3$ (Me_4Si as internal standard) or D_2O (CH_3CN as internal standard). Signal assignments such as 1^3 stand for a proton or carbon at C-1 of sugar redidue 3. **6**; ^{1}H ($CDCl_3$) δ 5.43 (bs, 1^1), 5.00 (d, 8.3 Hz, 1^2); ^{13}C: δ 98.36 (1^2), 85.06 (1^1). **7**; ^{1}H (D_2O) δ 5.39 (d, 1.5 Hz, 1^1), 4.45 (d, 8.4 Hz, 1^2); ^{13}C δ 99.41 (1^2), 85.59 (1^1), 70.30 (4^2). **9**; ^{1}H (D_2O) δ 5.46 (bs, 1^1), 4.53 (d, 8.1 Hz, 1^2), 4.40 (d, 7.6 Hz, 1^3), 1.91 (s, Me); ^{13}C δ 103.33 (1^3), 99.19 (1^2), 85.49 (1^1), 78.85 (4^2). **3**; ^{1}H ($CDCl_3$) δ 5.47 (bs, 1^1), 4.67 (d, 7.6 Hz, 1^2), 4.48 (d, 7.6 Hz, 1^3); ^{13}C δ 100.81 (1^2), 98.91 (1^2), 84.77 (1^1). **10**; ^{1}H ($CDCl_3$) δ 5.21 (bs, 1^1), 4.67 (m, Ser^{α}), 4.53 (d, 8.5 Hz, 1^2), 4.46 (d, 8.0 Hz, 1^3); ^{13}C δ 100.92 (1^3), 100.76 (1^2), 98.55 (1^1), 54.12 (Ser^{α}). **12**; ^{1}H (D_2O) δ 4.80 (bs, 1^1), 4.49 (d, 7.3 Hz, 1^2), 4.47 (m, Ser^{α}), 4.40 (d, 7.9 Hz, 1^3); ^{13}C δ 103.39 (1^3), 99.91 (1^2), 97.88 (1^1), 54.69 (Ser^{α}). **13**; ^{1}H (D_2O) δ 4.79 (bs, 1^1), 4.47(d, 7.7 Hz, 2H, 1^2 and 1^3), 4.44 (m, Ser^{α}), 2.69 (dd, 4.8 Hz, 3^{4e}), 1.96 (s, 6H, Me), 1.76 (t, 12.4 Hz, 3^{4a}); ^{13}C (^{t}BuOH as internal standard) δ 104.18 (1), 101.07 (1), 98.99 (1^1), 56.03 (Ser^{α}), 41.03 (3^4), 23.92, 23.66 (Me). **1**; ^{1}H (D_2O) δ 4.80 (bs, 1^1), 4.51 (d, 7.4 Hz, 1), 4.48 (d, 7.9 Hz, 1), 2.69 (dd, 4.5 Hz, 3^{4e}), 1.98, 1.96 (s, Me), 1.73 (t, 12.3 Hz, 3^{4a}).

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REVIEW

ACS chemical biology

Combined Approaches to the Synthesis and Study of Glycoproteins

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ABSTRACT Carbohydrates are the basis for many therapeutic and diagnostic strategies, yet the full potential of glycans in medicine has not been realized. The study of the precise role of different carbohydrates, bound to either proteins or lipids, is hampered by difficulties in accessing pure, well-defined glycoconjugates. This Review highlights recent advances in glycobiology with a particular emphasis on oligosaccharide synthesis and conjugation techniques for the construction of homogeneous glycoconjugates. New methods for the study of protein−glycan interactions such as carbohydrate arrays and *in vivo* visualization of glycosylation pattern changes will also be addressed. The development of glycotherapeutics is just beginning, and much remains to be understood about the relationship between glycoconjugate structure and function. The emergence of novel tools will certainly facilitate and expand the use of carbohydrates in therapeutics and diagnostics.

Carbohydrates constitute a large and diverse class of biopolymers. For a long time the primary role of carbohydrates in biology was viewed as a source of energy or as an integral part of cellular structure. However, over recent decades, it has become clear that the expression of complex carbohydrates in human cells is critical in the development and physiology of living systems (*1*–*4*). The mammalian glycome is thought to be complex, due to the inherent structural diversity of carbohydrate molecules (*5*). Nature uses this abundant repertoire of structures as specific codes in several biological processes such as cellular differentiation (*6*), regulation of cellular signaling (*7*), fertilization (*8*), and immune response (*9*). Unlike proteins and nucleic acids, glycoprotein biosynthesis is not under genetic control, resulting in heterogeneous mixtures—the so-called glycoforms (*2*). Complexity is further increased by competing pathways between different processing enzymes and substrates that result in complex, branched structures. Access to pure samples from natural sources is very challenging, despite some exceptions (*10*). It is also well-established that each component of these glycoforms may have different biological properties (*2*). Recent developments of recombinant glycosylation systems *in vivo* (*11*), oligosaccharide synthesis (*12*–*15*), site-selective protein glycosylation (*16*–*18*), glycoarrays (*19, 20*), and *in vivo* targeting of glycans (*21*) are leading to a better understanding of carbohydrate function in nature. However, the field of glycobiology still suffers from the lack of basic tools that fueled advances in genomics and proteomics. This Review focuses on recent advances in the synthesis of complex, biologically relevant carbohydrates, new methods for accessing well-defined glycoproteins and glycolipids, and new tools for analysis of glycosylation patterns and the study of glycan−protein interactions.

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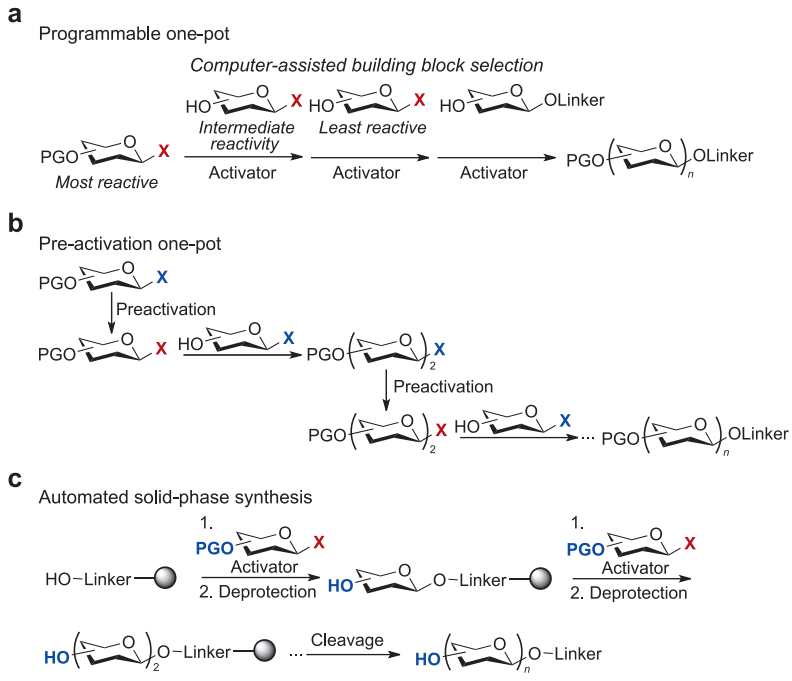
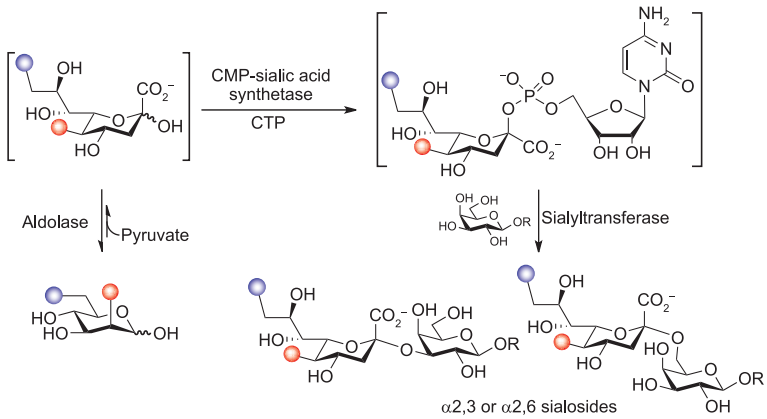
10.1021/cb900014n CCC: $40.75

Scheme 1. Oligosaccharide synthesis using (a) programmable one-pot synthesis, (b) pre-activation one-pot synthesis, and (c) automated solid-phase synthesis



Oligosaccharide Synthesis. The synthesis of oligosaccharides requires control over the stereochemistry of the glycosidic bond and regioselectivity. Furthermore, the number of glycan structures of interest is very large and thus commands a synthesis method that will allow a large-scale effort to make these glycans available (*22*). Recent advances in the assembly of oligosaccharides offer an unprecedented level of efficiency. Both chemical and chemoenzymatic synthesis have been improved. One-pot chemical synthesis streamlines the assembly of oligosaccharides (Scheme 1, panel a). The programmable one-pot synthesis relies on computer-assisted selection of building blocks with different activity of the anomeric leaving group based on the sugar and its protecting group pattern (*12*). This approach allows for the activation of the building block in the presence of the acceptor, which also possesses an anomeric leaving group. Successive addition of building blocks with decreasing reactivity and finally the reducing-end sugar completes the assembly of the protected oligosaccharide. The one-pot method requires one single purification step and removes the need for temporary protection of the acceptor alcohol. However, disaccharide building blocks are often needed for the assembly of structures above five residues. Protecting group pattern has to be tailored to the specific position of the monosaccharide in the oligosaccharide and increases the required number of building blocks. Preactivation protocols, where the anomeric leaving group is activated irreversibly, prior to the addition of the acceptor have addressed this shortcoming (Scheme 1, panel b) (*23*). The resulting disaccharide can be preactivated prior to the addition of the third sugar. Although preactivation introduces more flexibility in the protecting group scheme, the use of disaccharide buildings blocks is still necessary. The one-pot synthesis of a small library of oligosaccharide has been automated, but solution-phase chemistry will always be challenging to automate (*24*). A different approach to oligosaccharide assembly is based on automated solid-phase synthesis (Scheme 1, panel c) (*13, 25*). The method uses the successive glycosylation of a linker attached to a polymeric support

SCHEME 2. Chemoenzymatic oligosaccharide synthesis using a one-pot, three-enzyme system



with different monosaccharide building blocks. After assembly, the oligosaccharide is cleaved from the polymer support, purified, and deprotected. Automated solid-phase synthesis greatly simplifies the assembly of oligosaccharides and has the potential to make the glycan synthesis available for the nonexpert, as is the case for oligonucleotide and peptide synthesis. This method truly will be useful once all of the common glycosidic linkages are accessible. Thus, the focus of automated solid-phase synthesis has recently shifted to the synthesis of difficult glycosidic linkages such as α-galactose (*26*) and β-mannose (*27*). Different supports such as fluorous linkers have been explored (*28*) but have yet to deliver on the promise of automation.

Chemoenzymatic synthesis of oligosaccharides is often used as an alternative to existing chemical methods (*29–31*). The stereo- and regioselective glycosylation of unprotected glycans makes this approach particularly attractive. However, glycosyltransferases are not always available and require the use of expensive nucleotide-activated glycosides. *In vivo* synthesis and multienzyme systems have been used to prepare complex glycans on a large scale (*29, 32–36*). Recently, the discovery of highly active bacterial sialyltransferases has enabled the synthesis of sialosides in a one-pot, three-enzyme system starting from various mannosamines (Scheme 2) (*37, 38*). This method was used to generate a library containing natural and unnatural α2,3- and α2,6-sialosides. The challenge remains to identify other transferases with high activity and broad substrate specificity in order to gain access to unnatural glycans.

Carbohydrate Vaccines. The development of carbohydrate-based vaccines holds great promise for a host of diseases (*39*). Current strategies require conjugation of the low immunogenic carbohydrate antigen through a linker to a protein carrier for good antibody response (Figure 1). The protein carrier induces a potent T-cell response resulting in a cascade of cytokines that aid the antibody response against the protein carrier. In some cases good levels of carbohydrate-specific antibody were raised using conjugate constructs (*40–44*).

Despite these successes, serious challenges remain. Strong adjuvants often are required to induce a good immune response. High antibody titers against protein and linker can result in undesired immune suppression

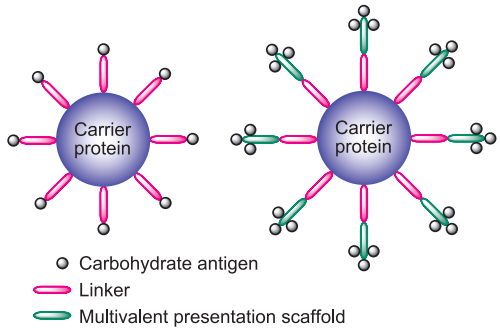


Figure 1. Typical glycoconjugate vaccine constructs.

of the carbohydrate epitope (*45, 46*). While methods for the efficient synthesis of pure antigenic carbohydrates are available, the construction of well-defined glycoconjugates as vaccines is rare. Methods used for the conjugation of the carbohydrate antigen to a protein carrier generally rely on the alkylation of nucleophilic side chains of lysine and cysteine residues (*47*). Lack of chemoselectivity generates highly polydisperse mixtures that are the basis for immunological studies. The efficacy of such constructs in generating antibodies cannot be attributed definitely to a single antigen copy number. Mass spectrometry analysis typically reveals a statistical mixture of carbohydrate antigen copies. Recently, a robust immune response was generated using a *single* copy of a carbohydrate epitope coupled to a synthetic peptide (*48*). This is one of only a few, successful examples of a well-defined glycoconjugate construct used in vaccination (*49–51*). This result encourages the continued investigation of homogeneous constructs in vaccination studies. A coherent strategy that coordinates both carbohydrate synthesis and conjugation methodology allowing for the construction of uniform protein conjugates is highly desirable. Structure−activity relationships and thus glycoconjugate structure and immunogenicity could be correlated.

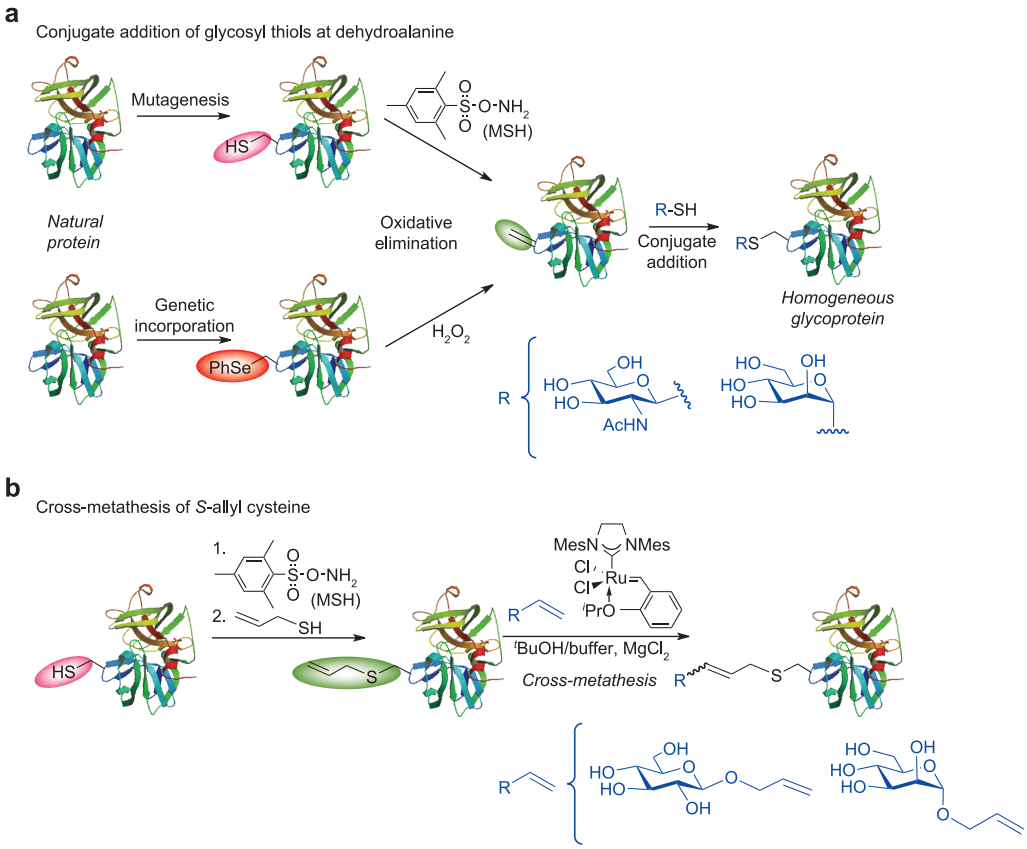
Glycoconjugation Methods. Recent advances in the site-selective modification of proteins enables the creation of pure, well-defined glycoconjugates to study the role of glycans and for applications such as vaccination (*16–18*). One approach that has been explored to avoid selectivity issues is to introduce a non-native amino acid that contains a side chain with a bio-orthogonal functionality. Chemical ligation at that specific site gives access to homogeneous modified proteins. Recently, access to dehydroalanine-containing proteins and its use as a Michael acceptor for glycosyl thiols has been demonstrated (Scheme 3, panel a). A novel, efficient oxidative-elimination reaction of cysteine with *O*-mesitylenesulfonylhydroxylamine (MSH) gives access to dehydroalanine (*52*). Alternatively, incorporation of phenyl selenocysteine into a protein through misacylated tRNAs can be converted to dehydroalanine upon treatment with peroxide (*53*). In both cases, dehydroalanine proved to be an efficient chemical handle to access glycosylated cysteine derivatives. Previous studies on the desulfurization of a disulfide-linked glycoprotein had enabled the first conversion of cysteine to a thioether linked glycoprotein (*54*). Access to glycosylated proteins was also shown to be possible using olefin metathesis (*55*). Allyl sulfides are privileged substrates for aqueous cross-metathesis that are easily installed at dehydroalanine, allowing for the conjugation of carbohydrates to proteins (Scheme 3, panel b) (*55*). This conjugation methodology might prove useful in the development of glycoproteins for vaccination purposes when combined with automated procedures for the synthesis of carbohydrate antigens that often bear a terminal alkene.

Three-dimensional structural investigations of glycoproteins have been hampered by the heterogeneous nature of the available samples. Recently, a new NMR-based method for the study of glycoproteins has been demonstrated (*56*). Sequential labeling and *in vitro* *N*-glycosylation was used to access a nonlabeled glycan attached to a uniformly labeled protein in milligram amounts. The glycosylation acceptor site as well as the glycan structure of *Campylobacter jejuni* glycoprotein was fully characterized using this strategy.

Chemoenzymatic methods have been successfully employed for the construction of glycoproteins (*57*). This approach involves preparation of a homogeneous glycoprotein acceptor by either selective deglycosylation of natural or recombinant proteins or by modern chemical protein synthesis techniques. Subsequent stereo- and regiospecific enzyme-catalyzed transglycosylation gives access to complex, well-defined glycoproteins (*57*). Incorporation of a ketone handle into a protein followed by reaction with a *N*-acetylglucosamine (GlcNAc) derivative containing a hydroxylamine reactive group results in a GlcNAc oxime-linked glycoprotein (*58*). Sequence-specific and differential enzymatic elaboration at the previously installed GlcNAc site was shown (*58*). More recently, endo A-catalyzed transglycosylation (*59, 60*) has been applied to the synthesis of complex glycoproteins (*61, 62*). Importantly, assembly of the native core *N*-pentasaccharide ($Man_3GlcNAc_2$) and further elaboration is possible using this method (Scheme 4) (*62*). Combined yeast expression and *in vitro* chemoenzymatic glycosylation was used to assemble human IgG1-Fc (*63*). These examples illustrate the power of combined chemical and enzymatic methods.

Many proteins are attached to cell surfaces *via* a glycosyl phosphatidylinositol (GPI)-anchor. Native chemical ligation (*64*) (NCL) was employed to ligate a synthetic, cysteine-tagged GPI anchor with recombinant prion protein (PrP) bearing a C-terminal thioester in the

SCHEME 3. Chemical site-selective protein glycosylation



first example for homogeneous GPI-anchored PrP (Figure 2) (*65*). Access to this important class of post-translationally modified proteins provides the basis for a detailed analysis of the influence of GPI anchors on protein structure and function. NCL has often been used for the construction of well-defined glycoproteins (*66*). For example, the glycoprotein ribonuclease C has been accessed using this strategy (*67, 68*). Importantly, the semisynthetic enzyme displayed activity comparable to that of natural enzyme.

Novel Tools for Carbohydrate Research. Carbohydrates are abundantly displayed on the surface of cells with implications in various physiological and pathological processes (*4*). The surface of malignant cells displays a characteristic aberrant glycosylation pattern (*69*). The ability to explore the biological information content of carbohydrates, attached to either proteins or lipids, has become a primary focus of glycomics research.

Probing Glycosylation. Real-time visualization of changes in glycosylation patterns in cells and living animals is now possible (*21, 70*). Metabolic incorporation of a non-native carbohydrate bearing a bio-orthogonal functional group that acts as a chemical reporter into the cell biosynthetic machinery initiates the process. The modified glycan

KEYWORDS

Automated synthesis of oligosaccharides: Programmed synthesis of oligosaccharide structures by a machine.

Bio-orthogonal: Non-native chemical handle that allows chemical modification without compromising the protein solution or living cell.

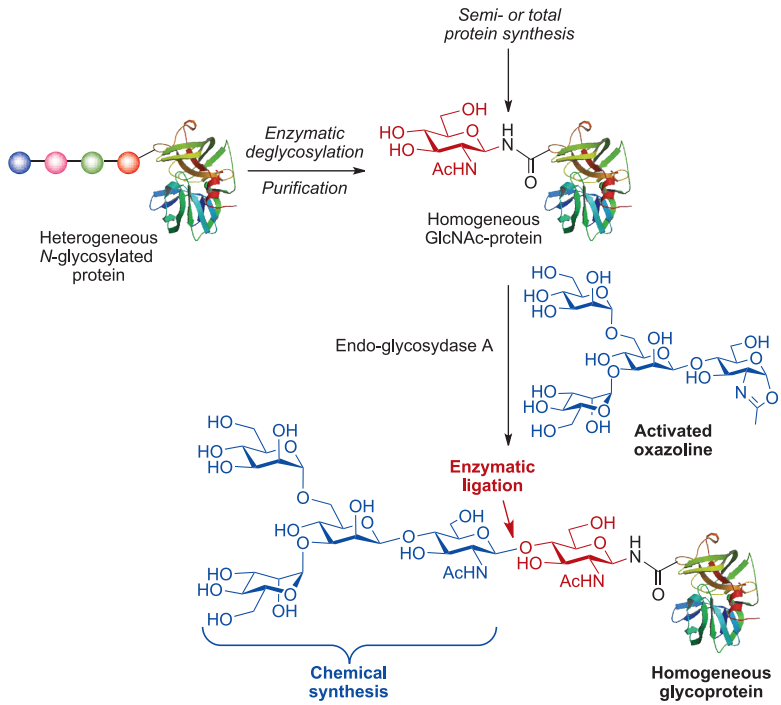
Chemoenzymatic: Combined chemical and enzymatic approach toward the synthesis of biomolecules.

Glycome: Entirety of glycans associated within an organism.

Glycoarrays: Glycans attached to a surface in a spatially defined and miniaturized fashion.

Glycoform: Glycosylated proteins that possess the same peptide backbone but different nature and site of glycosylation.

Scheme 4. Combined chemical and enzymatic approach for the synthesis of homogeneous *N*-glycoproteins



is then processed and incorporated on the cell surface. Subsequent reaction with a detectable probe equipped with a complementary bio-orthogonal functional group enables detection of the incorporated non-native glycan. The chemical reporter is metabolically stable and inert in biological settings and selectively reacts with phosphines to generate an aza-ylide intermediate that, upon hydrolysis, gives an amine and phosphine oxide—Staudinger ligation (*71*) and alkynes in a [3 + 2] Cu(I)-promoted cycloaddition resulting in a triazole

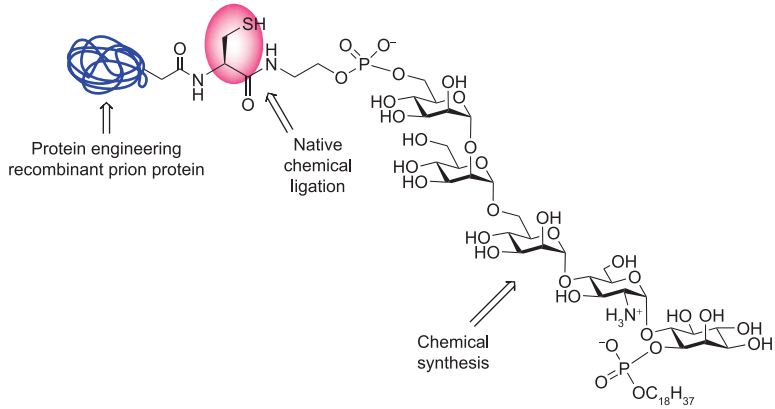


Figure 2. Synthesis of a semisynthetic, homogeneous GPI prion protein (PrP).

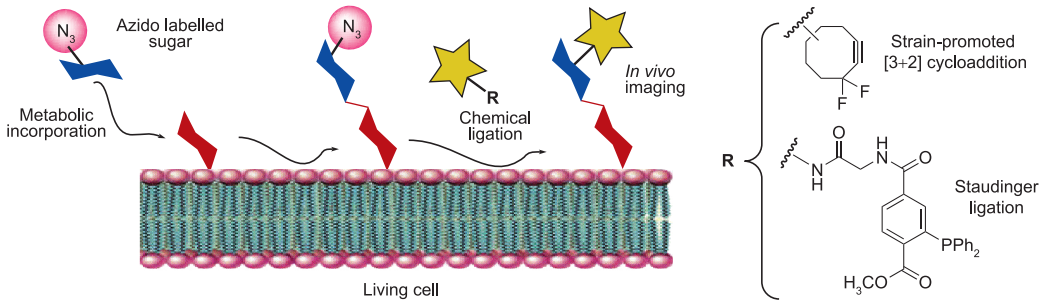


Figure 3. Schematic view of the use of azido-labeled sugars for carbohydrate imaging in living systems.

(*72, 73*). These two reactions are now standard tools in chemical biology, with the Staudinger ligation finding extensive use to probe *in vivo* glycosylation in cells (*74, 75*), allowing, for example, identification of *O*-GlcNAc proteins (*76*). More recently, a strain-promoted [3 + 2] cycloaddition between azides and cyclooctyne that does not require cytotoxic copper has been disclosed (Figure 3) (*77, 78*). This methodology was successfully used for *in vivo* imaging of membrane-associated glycans in developing zebrafish embrios (*77*). 4-Dibenzocyclooctinols are also efficient reagents in copper-free Huisgen cycloaddition reactions allowing for real-time monitoring of glycan trafficking in living cells (*79*).

Carbohydrate Arrays. System-wide analysis techniques such as DNA and protein arrays have benefited genomics and proteomics. Glycobiology lacked routine tools for glycan profiling until carbohydrate arrays emerged as the high-throughput analytical technique of choice for the study of glycan–protein interactions, starting in 2003 (*80, 81*). Chip-based glycan arrays enable screening of several thousand binding events in a single read-out. Importantly, this technique requires very little glycan sample. The presence of a linker between the glass surface and the sugar ensures presentation of the glycan to the binding partner. The diverse display of glycans on a single chip mimics glycan presentation on cells allowing multivalent interactions of relatively weak protein–glycan interactions. These interactions are generally detected by the use of fluorophore-labeled proteins or by antibody incubation (*19, 20*). Surface plasmon resonance (SPR) allows for label-free analysis of protein–carbohydrate interactions on microarrays (*82*).

Carbohydrate arrays rapidly became the tool of choice for the study of glycans in systems biology (*83*). Carbohydrate arrays have found most use in the efficient screening of carbohydrate-binding proteins (*19, 20*). Focused carbohydrate microarrays complement the Consortium for Functional Glycomics (USA) general carbohydrate array. These include, for instance, host specificity of avian and human influenza strains (*84*) or epitope mapping of HIV- and tumor-associated antibodies (*85–87*). Heparin microarrays aided the identification of specific sequences recognized by different fibroblast growth factors (*88*), and serologic search for autoimmune disease antibodies led to the discovery of novel Crohn's disease markers (*89*). Immobilization of sulfated chondroitin tetrasaccharides led to the identification of novel tumor necrosis factor-α antagonist (*90*). Enzymatic elaboration of immobilized glycans is also possible (*91*). Additionally, kinetic constants can be calculated from binding intensities at different dilutions (*92, 93*). More recently, covalently immobilized synthetic GPI glycans were used to assess the level and specificity of anti-GPI antibodies in individuals exposed to malaria disease and naïve individuals (Figure 4) (*94*). Additionally, phosphatidylinositol mannosides (PIMs)—essential precursors of more complex mycobacterial cell wall glycolipids—have been synthesized and immobilized in

KEYWORDS

Glycotools: Toolkit for the synthesis, conjugation, and analysis of carbohydrates in biological systems.

Carbohydrate-based vaccines: Carbohydrate antigen based vaccines; typically the carbohydrate antigen is conjugated to a protein carrier.

GPI anchored protein: Protein modified posttranslationally by glycosylation and lipidation; glycosyl phosphatidylinositol (GPI) anchor is used for the correct attachment of proteins to cell surfaces.

Native chemical ligation (NCL): widespread method for the total synthesis of proteins based on seminal ligations developed by Wieland and co-workers in 1953 (*98*); involves the reaction of two unprotected peptide moieties bearing a C-terminal thioester and a N-terminal cysteine.

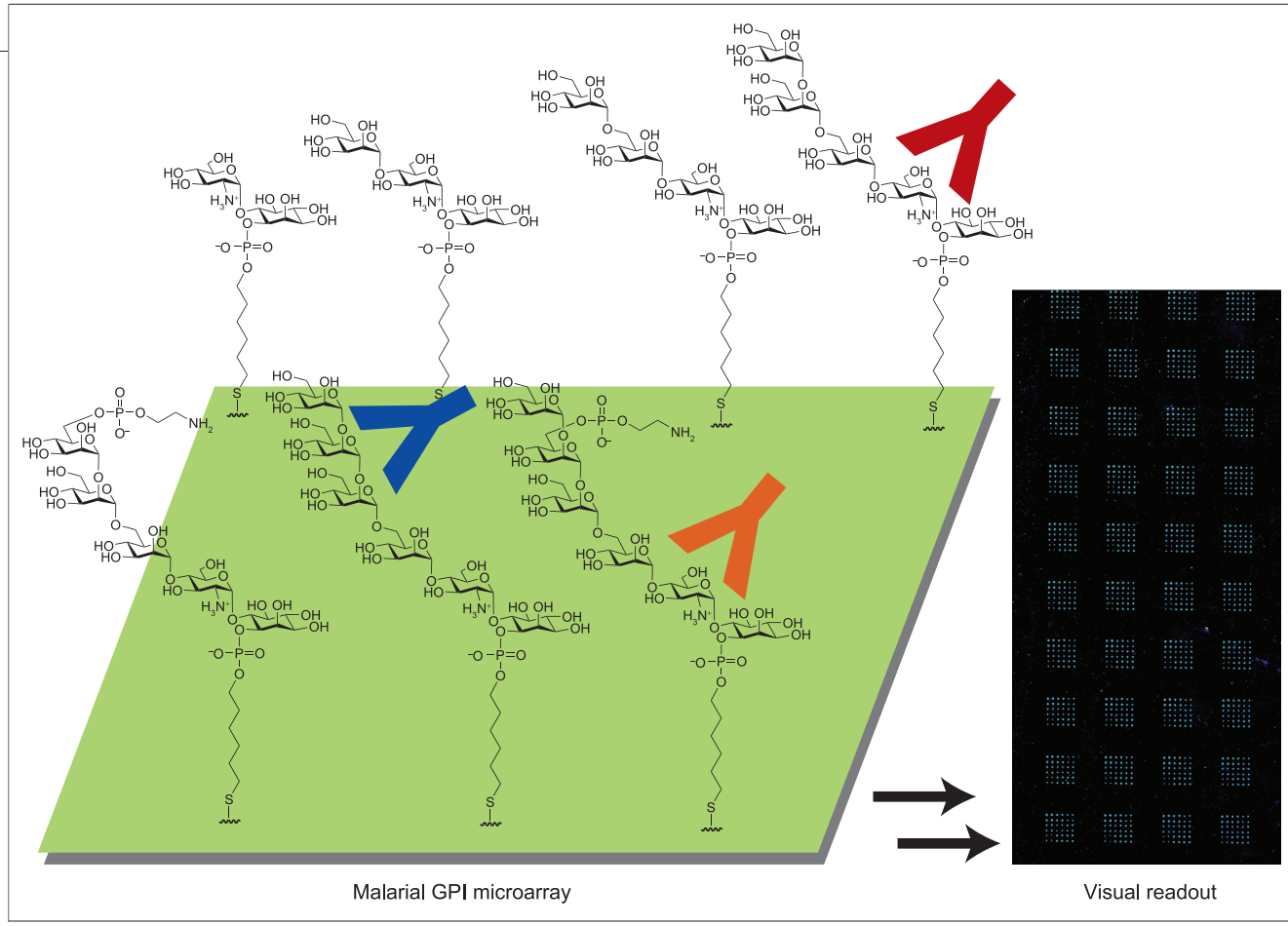


Figure 4. Malarial GPI microarray to probe anti-GPI antibody levels and specificity. Use of covalently bound chemically synthesized GPI on a glass surface to specifically analyze anti-GPI malarial antibodies of noninfected and infected serum.

microarray slides to elucidate differences in binding to the dendritic cell-specific intercellular adhesion molecule grabbing nonintegrin (DC-SIGN) receptor (*95*). Immobilized lectin arrays have been used to identify the presence of certain glycan structures in a glycoprotein sample (*96*). This methodology was further applied in the analysis of a dynamic bacterial glycome (*97*).

Carbohydrate array technology is an important tool that will play a critical role in the correlation of the genome and proteome with the glycome of an organism.

Conclusions. Glycan-based therapies hold great promise. However, realizing the full potential of glycans in medicine has been hampered by difficult access to glycoconjugates and the lack of tools for the study of the biological role of these biopolymers. While recent advances in oligosaccharide synthesis, glycoconjugation methodologies, and array and biochemical techniques for glycan profiling have reduced barriers in glycobiology, the glycotools reviewed here are still far from methods available for proteomic and genomic research. Glycomics will certainly benefit from continued efforts in developing glycan technologies. These tools will not only bring deeper insights into the molecular mechanisms but also result in greater advances into their use in therapeutics and diagnostics. We anticipate that automated synthesis in combination with novel glycoconjugation techniques will allow for the construction of well-defined glycoproteins for vaccination, with the design of such vaccines greatly benefiting from the use of glycoarrays for epitope profiling. For three recent publications of considerable relevance to this topic, see refs *99*–101.

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Oligosaccharide Synthesis with Glycosyl Phosphate and Dithiophosphate Triesters as Glycosylating Agents

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Abstract: Described is an efficient one-pot synthesis of α- and β-glycosyl phosphate and dithiophosphate triesters from glycals via 1,2-anhydrosugars. Glycosyl phosphates function as versatile glycosylating agents for the synthesis of β-glucosidic, β-galactosidic, α-fucosidic, α-mannosidic, β-glucuronic acid, and β-glucosamine linkages upon activation with trimethylsilyl trifluoromethanesulfonate (TMSOTf). In addition to serving as efficient donors for *O*-glycosylations, glycosyl phosphates are effective in the preparation of *S*-glycosides and *C*-glycosides. Furthermore, the acid-catalyzed coupling of glycosyl phosphates with silylated acceptors is also discussed. Glycosyl dithiophosphates are synthesized and are also used as glycosyl donors. This alternate method offers compatibility with acceptors containing glycals to form β-glycosides. To minimize protecting group manipulations, orthogonal and regioselective glycosylation strategies with glycosyl phosphates are reported. An orthogonal glycosylation method involving the activation of a glycosyl phosphate donor in the presence of a thioglycoside acceptor is described, as is an acceptor-mediated regioselective glycosylation strategy. Additionally, a unique glycosylation strategy exploiting the difference in reactivity of α- and β-glycosyl phosphates is disclosed. The procedures outlined here provide the basis for the assembly of complex oligosaccharides in solution and by automated solid-phase synthesis with glycosyl phosphate building blocks exclusively or in concert with other donors.

Introduction

The role of carbohydrates in many biological pathways has become more defined in recent years. The traditional view of carbohydrates as solely sources of energy has been augmented by advances in glycobiology that establish oligosaccharides and glycoconjugates as essential components of information transfer in biological systems.[1] Specific oligosaccharides that participate in both beneficial and pathogenic events have been identified. Oligosaccharide components of human milk are known to protect breast-fed infants from a host of bacterial infections.[2] On the other hand, the cell-surface glycoconjugates found on protozoan parasites (e.g. *Leishmania*) serve to infect human hosts.[3] Finally, particular oligosaccharides such as the globo H hexasacccharide indicate malignant transformation of human breast, prostate, or ovarian cancer cells.[4] A better understanding of the biological capacity of oligosaccharides will eventually lead to the development of novel therapeutics and nutritional supplements targeting these interactions.[5]

The limited availability of complex oligosaccharides remains a major impediment to the study of carbohydrates. The purification of glycoconjugates from natural sources is in most cases extremely difficult due to the microheterogeneity of this class of biopolymers and is practical only on a very small scale (microgram to milligram). The absence of amplification techniques equivalent to the polymerase chain reaction (PCR) that revolutionized nucleic acid research further complicates matters. Currently, there are two synthetic methods available for the preparation of oligosaccharides and glycoconjugates as research tools; however, many challenges remain. Besides the traditional chemical techniques, enzymes have seen more frequent use due to their specificity and efficiency. Enzymatic oligosaccharide synthesis has been scaled up to produce kilogram quantities of complex carbohydrates.[6] While attractive for production on a commercial scale, a shortcoming of this method is the narrow scope of substrates accepted by the enzymes and the need to have access to all glycosyl transferases involved in the preparation of a particular sequence.

Synthetic chemists have been addressing the challenges associated with the preparation of complex carbohydrates for over one hundred years. During this time, numerous versatile building blocks that function as efficient glycosyl donors have been developed.[7] While glycosyl trichloroacetimidates[8] and thioglycosides[9] are the most commonly used methods of glycosylation, glycosyl sulfoxides,[10] *n*-pentenyl glycosides,[11] glycosyl phosphites,[12] glycosyl halides,[13] and anhydrosugars[14] all see wide-

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spread use. The need for new, readily synthesized, stable, and highly reactive glycosylating agents still persists.

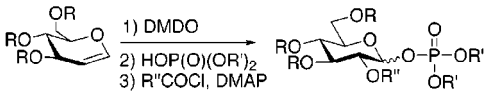
Nucleotide 5′-diphospho sugars (NDPs) serve as substrates for the glycosyl transferases that are responsible for the biosynthesis of oligosaccharides and may thus be considered nature's building blocks.[15] Several approaches to the synthesis of glycosyl phosphates in the form of glycosyl 1-phosphates and NDPs had been reported previously.[16,17] Despite the variety of methods available for the synthesis of glycosyl phosphate mono-, di-, and triesters, the use of these phosphates, and their sulfur analogues, in the chemical synthesis of oligosaccharides has received little attention.[18]

Here, we describe the convenient synthesis of glycosyl phosphate[19] and glycosyl dithiophosphate[20] triesters from glycal precursors. The one-pot synthesis of glycosyl phosphates and derivatives can be performed in high overall yield on a multigram scale and requires minimal chromatographic purification. A panel of activation conditions for these novel glycosylating agents was explored and glycosyl phosphates were found to function as efficient donors for the synthesis of *O*-, *S*-, and *C*-glycosides. Taking advantage of inherent selectivity of glycosyl phosphate mediated reactions, we developed a regioselective glycosylation strategy. Also, an orthogonal glycosylation method involving the activation of phosphate donors in the presence of thioglycosides provides a two-step synthesis of a trisaccharide without the need for intermediate protecting group manipulations. Finally, a unique approach to controlling reactivity of glycosylation reagents by exploiting the inherent reactivity difference between α- and β-phosphates is introduced. This method was successfully applied to the synthesis of a trisaccharide. The procedures outlined here provide the basis for the assembly of complex oligosaccharides in solution and by automated solid-phase synthesis using glycosyl phosphate building blocks.

Results and Discussion

Glycosyl Phosphates. Glycals are attractive starting materials for the preparation of differentially protected building blocks that serve in oligosaccharide assembly. In contrast to other hexoses, glycals possess only three hydroxyl groups that require differentiation by introduction of protective groups when compared to five hydroxyl groups in fully oxygenated sugars. The glycal assembly method has recently been exploited in the synthesis of glycosylated natural products and numerous oligosaccharides both in solution and on the solid support.[14] In the course of these studies it had been demonstrated that other glycosylating agents, such as thioethyl glycosides, can be prepared from glycals.[21] The need for excess thiol reagents and the modest yields of this transformation prompted us to explore the synthesis of other isolable glycosylating agents from glycal precursors.

Scheme 1. Synthesis of Glycosyl Phosphates



Initially we investigated a one-pot procedure for the convenient synthesis of glycosyl phosphates using phosphoric acid diesters in the ring opening of 1,2-anhydrosugars (Scheme 1).[16g,19] Commercially available dibutyl and dibenzyl phosphate diesters quantitatively provided C2−OH glycosyl phosphate triesters[22] upon reaction with 1,2-anhydrosugars at −78 °C. In most cases, glycal epoxidation was complete in 5 min (Figure 1).[23] In situ acylation of the newly generated C2 hydroxyl group afforded fully protected glycosyl phosphates.

Interestingly, when the epoxide opening was carried out in dichloromethane or toluene, β-glycosyl phosphates were obtained with high selectivity. Conversely, the α-glycosyl phosphates predominated when tetrahydrofuran was used as a solvent for the opening of the epoxide. The observed solvent effect was rationalized through the finding that anomerization takes place more rapidly in tetrahydrofuran (Scheme 2). For example, when the ring-opening of 1,2-anhydrosugar **11a** was carried out in CH_2Cl_2 followed by acylation, only β-phosphate **11** was obtained (Scheme 2). Similarly, when the ring-opening was performed in THF and the newly generated C2−OH was immediately acylated, only the β-phosphate was obtained. When the ring-opening was allowed to stir at ambient temperature for 8 h and then acylated, a 1:1 mixture of anomers **11** and **12** was obtained. These results support earlier findings that α-phosphates could be formed from the β-isomers by acid-catalyzed anomerization. The possibility of creating different types of anomeric phosphates proved particularly important with respect to the glycosylation properties of the ensuing species, as discussed below.

The installation of C2 protecting groups other than esters proved challenging. Benzylation employing sodium hydride and benzyl bromide resulted in migration of the phosphate to yield the C2-phosphoryl benzyl glycoside. Milder benzylation conditions involving benzyl bromide/silver(I) oxide also did not meet with success and the incorporation of silyl groups using silyl triflates in the presence of 2,6-lutidine led to phosphate decomposition. Triethylsilyl ethers, on the other hand, were readily prepared by reaction of the C2-hydroxyl group of the glycosyl phosphate with triethylsilyl chloride and imidazole in DMF.

Previous reports of the instability of glycosyl phosphates toward silica gel column chromatography prompted us to simplify the purification of the reaction products.[16d,f] Precipitation of unwanted byproducts was accomplished by the addition

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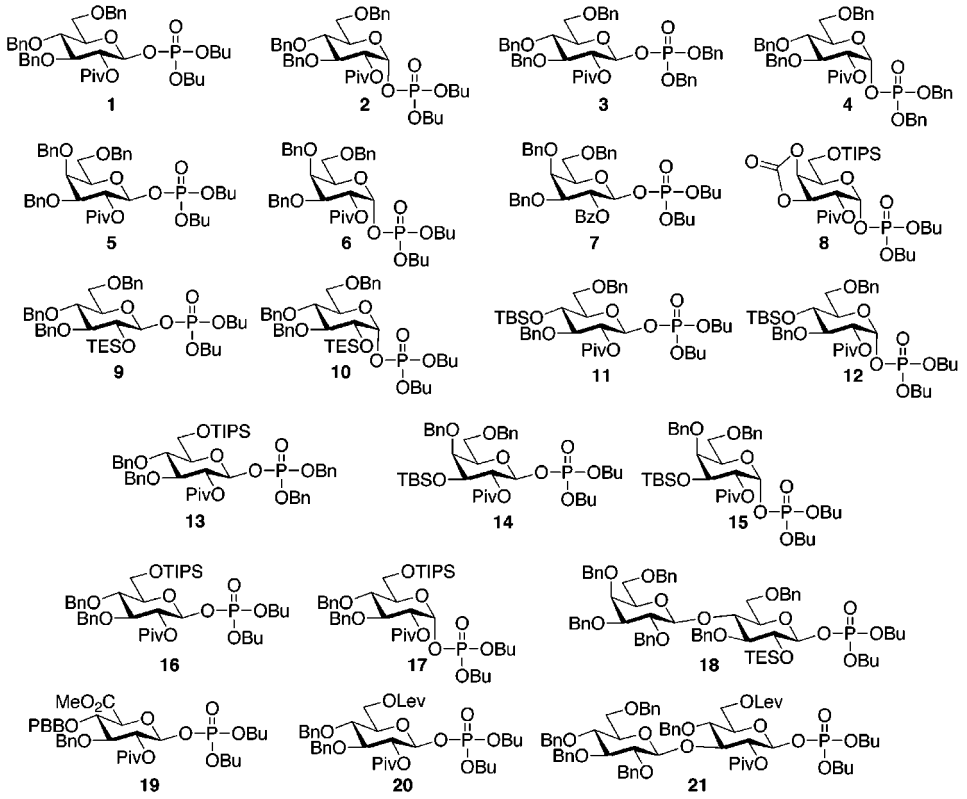
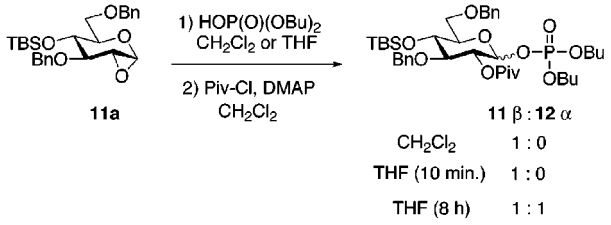


Figure 1. Glycosyl phosphates prepared from glycals.

Scheme 2. Solvent Effect on 1,2-Anhydrosugar Ring-Opening

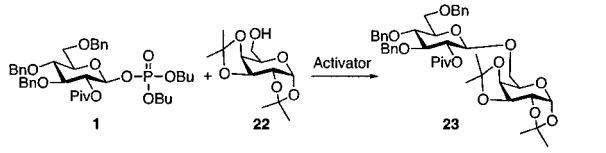


of ethyl acetate/hexane mixtures to the reaction mixture following acylation. Filtration through a pad of silica was sufficient to yield pure glucosyl and galactosyl phosphates (Figure 1). In pure form, α- and *β*-glycosyl phosphates were found to be completely stable to storage for several months at 0 °C.

With a straightforward procedure for the synthesis of gram quantities of glycosyl phosphates in hand, we explored their use as glycosyl donors under the agency of various activators. Trimethylsilyl triflate (TMSOTf) had been reported to activate glycosyl phosphates,[18a] but a thorough analysis of other potential activating reagents had not been described. Screening of a variety of Lewis acids revealed that tin(II) chloride, zinc iodide, zinc triflate, and copper(II) triflate have moderate activity for glycosyl phosphate activation, although product formation was accompanied by side-products (Figure 2). Notably, the silyl triflate reagents TMSOTf and TBSOTf ensured high-yielding glycosylations while the use of BF_3-OEt_2 offered modest results.

Next, the scope of glycosylation reactions employing glycosyl phosphates was explored (Table 1). Glucosyl and galactosyl *β*-phosphates bearing C2-participating groups reacted rapidly with primary and hindered secondary hydroxyls. Whereas the glycosyl *β*-phosphates were sufficiently reactive at −78 °C, the more stable glycosyl α-phosphates, such as **2**, served as competent glycosyl donors only at higher temperatures (−20 → −40 °C). In exploring novel glycosylating agents, it was important for us to establish that linkages commonly encountered in biologically relevant structures can be accessed in good yield. Creation of galactose *β*-(1→4) glucosamine glycosidic linkages common to several Lewis blood group determinants was readily accomplished by coupling of galactosyl phosphate **7** and glucosamine **27**. The efficient glycosylation of electronically and sterically challenging substrates such as **24** and **27** demonstrates the utility of glycosyl phosphates in oligosaccharide synthesis. In addition to their utility for glycoside formation in solution phase, glycosyl phosphates have also proven useful under the solid-phase paradigm.[20] Differentially protected glycosyl phosphates **20** and **21** have been recently used in the automated synthesis of phytoalexin elicitor *β*-glucans.[24]

Deactivated donors such as glucuronic acid phosphate **19** were found to be highly efficient in reactions with primary or

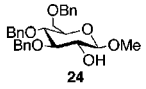
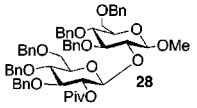
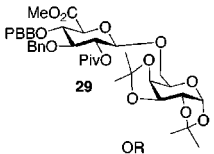
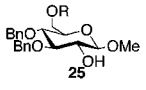
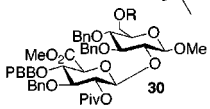
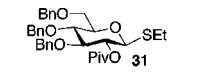
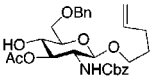
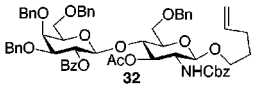
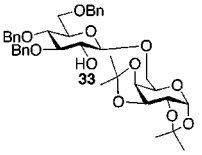


Activator[a]	Solvent	Temp. (C)	Time (h)	Yield (%)
TMSOTf	CH_2Cl_2	-78°	0.25	94
TBSOTf	CH_2Cl_2	-78°	0.25	95
BF_3-OEt_2	CH_2Cl_2	-78°	0.5	81
$SnCl_2$	CH_2Cl_2	0° → rt	5	61
ZnI_2	CH_2Cl_2/THF	0° → rt	5	30

Figure 2. Reagents screened for the activation of glycosyl phosphates. Footnote a: Other activators that were examined but showed no productive couplings included $Mg(OTf)_2$, $SnCl_4$, $TiCl_4$, $ZnCl_2$, $MgCl_2$, $CuCl_2$, $ZrCl_4$, $LaCl_3$, $FeCl_2$, $MgBr_2$ OEt_2, $SnCl_2/AgClO_4$, $MnCl_2$, and CSA.

(24) Plante, O. J.; Palmacci, E. R.; Seeberger, P. H. *Science* **2001**, *291*, 1523.

Table 1. Glycosylations with Glycal Derived Glycosyl Phosphates[a]

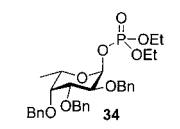
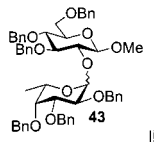
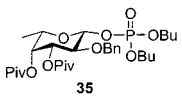
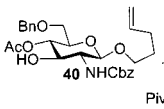
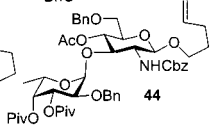
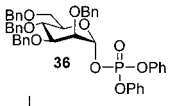
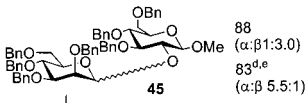
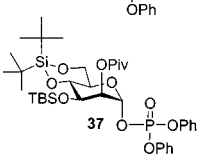
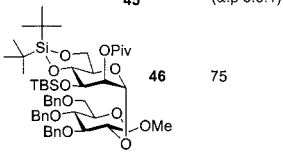
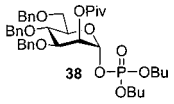
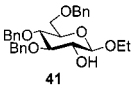
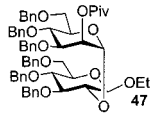
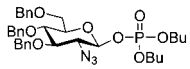
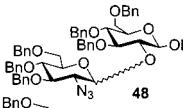
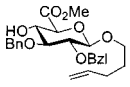
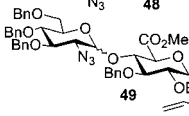
Donor	Acceptor	Product	Yield (%)[a]
1	 **24**	 **28**	83
2	**22**	**23**	87[b]
19	**22**	 **29**	72[b]
19	 **25**	 **30**	84[b,d]
3	EtSH **26**	 **31**	90
7	 **27**	 **32**	96[c]
9	**22**	 **33**	71

[a] All reactions were carried out under the following conditions unless otherwise noted: 1.2 equiv of donor, 1.0 equiv of acceptor, 1.2 equiv of TMSOTf in CH_2Cl_2 at −78 °C for 30 min. [b] −20 °C for 30 min. [c] 1.0 equivof acceptor, 1.7 equiv of donor, and 1.7 equiv of TBSOTf, −50 → −20 °C for 30 min. [d] R = α-azidomethyl benzoate.

secondary alcohols (Table 1).[25] These findings are particularly noteworthy because biologically important polysaccharides including glycosaminoglycans such as heparin and chondroitin are composed of alternating uronic acid and glucosamine monomers. Combined with the straightforward synthesis from readily accessible glucuronic acid glycal precursors, the use of **19** as a glycosylating agent provides a direct entry to complex glycan structures.

The high reactivity observed with the glucose, galactose, and glucuronic acid donors prompted us to explore the properties of glycosyl phosphates prepared from sugars such as fucose, mannose, and glucosamine. Several protocols for the synthesis of these glycosyl phosphates via phosphorylation of lactol precursors were available.[16] Fucosyl phosphate **34** and mannosyl phosphates **36** and **37** were prepared from the corresponding lactols and phosphochloridates in the presence of *N*,*N*-(dimethylamino)pyridine (DMAP).[16d,26,27] The synthesis of glucosamine donor **39** and fucose donor **35** proved more challenging and these glycosylating agents were obtained most efficiently from the anomeric trichloroacetimidates.[16b] Mannosyl phosphate **38** was obtained from the mannosyl 1,2-anhydrosugar that was

Table 2. Glycosylations with Fucosyl, Mannosyl, and Glucosamine Phosphates[a]

Donor	Acceptor	Product	Yield (%, α:β)[a]
 **34**	**24**	 **43**	95[b] (α:β 3:2)
 **35**	 **40** NHCbz	 **44**	97[c]
 **36**	**24**	 **45**	88 (α:β1:3.0) 83[d,e] (α:β 5.5:1)
 **37**	**24**	 **46**	75
 **38**	 **41**	 **47**	82
 **39**	**24**	 **48**	77[d] (α:β 4:1) 61[d,e] (α:β 4:1)
39	 **42**	 **49**	60[d] α:β 1:2.5 60[d,e] α:β 1:1

[a] All reactions were carried out under the following conditions unless otherwise noted: 1.2 equiv of donor, 1.0 equiv of acceptor, 1.2 equiv of TMSOTf in CH_2Cl_2 at −78 °C for 30 min. [b] 1.0 equiv of acceptor, 1.5 equiv of donor, 1.6 equiv of TMSOTf at −20 °C for 10 min. [c] 1.0 equiv of acceptor, 3.0 equiv of donor, 3.0 equiv of TMSOTf −78 → −40 °C. [d] Reaction was performed at −40 °C. [e] CH_3CN was used as the solvent.

derived from 2-*O*-acetyl-mannosyl chloride upon treatment with KO*t*-Bu.[28]

Glycosylations involving the use of donors **34**−**39** are outlined in Table 2. Initially, perbenzylated fucose phosphate **34** was employed with a C2 glucosyl acceptor to determine the degree of stereoselectivity in the absence of participating groups. Activation of **34** at −20 °C furnished coupled product in nearly quantitative yield as an inseparable mixture of diastereomers (95% $\alpha:\beta$ = 3:2) as determined by ^{1}H NMR integration. Previously, glycosylation with other fully benzylated fucose donors (bromides,[29] trichloroacetimidates,[8] fluorides,[30] thioglycosides,[31] and *n*-pentenyl glycosides[32]) had also been reported to give α/β mixtures. While we were encouraged by the high yield of this reaction, a fucosylation method furnishing exclusively the biologically relevant α-linked glycosides was desirable. Donor **35**, bearing C3 and C4 ester groups, allowed for completely α-selective fucosylation in excellent yield (97%). Even though the preparation of fucose donors incorporating

(25) For use of the *p*-bromobenzyl (PBB) protecting group see: Plante, O. J.; Buchwald, S. L.; Seeberger, P. H. *J. Am. Chem. Soc.* **2000**, *122*, 7148.

(26) For prior syntheses of fucosyl phosphates see: (a) Duynstee, H. I.; Wijsman, E. R.; van der Marel, G. A.; van Boom, J. H. *Synlett* **1996**, 313. (b) Nunez, H. A.; O'Connor, J. V.; Rosevear, P. R.; Barker, R. *Can. J. Chem.* **1981**, *59*, 2086.

(27) (a) Hung, S.-C.; Wong, C.-H. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 2671. (b) Plante, O. J.; Palmacci, E. R.; Seeberger, P. H. *Org. Lett.* **2000**, *2*, 3841.

(28) Du, Y.; Kong, F. *J. Carbohydr. Chem.* **1995**, *14*, 341.

(29) Dejter-Juszynski, M.; Flowers, H. M. *Carbohydr. Res.* **1971**, *18*, 219.

(30) Nicolaou, K. C.; Hummel, C. W.; Bockovich, N. J.; Wong, C.-H. *J. Chem. Soc., Chem. Commun.* **1991**, *10*, 870.

(31) Lonn, H. *Carbohydr. Res.* **1995**, *139*, 105.

(32) Udodong, U. E.; Srinivas, R. C.; Fraser-Reid, B. *Tetrahedron* **1992**, *48*, 4713.

participating C4-ester groups requires longer synthetic routes than perbenzylated donors, two characteristics are noteworthy. Selective α-fucosylations can be effected due to a proposed long-range participation of the C4-ester and the resulting α-product is less prone to hydrolysis under acidic conditions when electron-withdrawing groups are present on the fucose ring.[33]

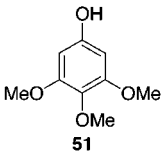
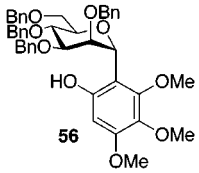
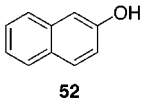
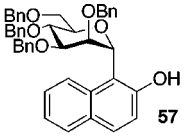
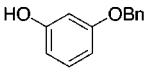
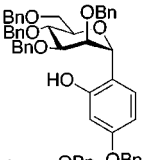
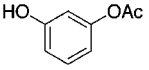
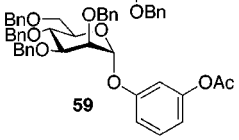
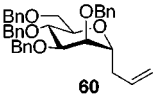
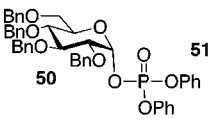
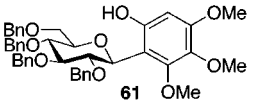
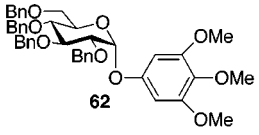
In addition to fucose donors, we were interested in exploring the properties of mannose donors carrying participating and nonparticipating groups on the C2 hydroxyl. Ubiquitous in nature, α-mannosides are major constituents of lipophosphoglycans and are, along with *β*-mannosides, integral components of *N*-linked glycoproteins. Mannose donor **37**, equipped with a C2 participating group, was used in the synthesis of α-mannosides and proved to be a reliable and efficient donor. Depending on the choice of solvent both α- and *β*-enriched mannosides were accessed with perbenzylated donor **36** (Table 2).[34] Coupling of **36** and secondary alcohol **24** preferentially afforded α-mannoside **45** when the reaction was carried out in acetonitrile (*β*:α = 1:5.5). Interestingly, a reversal of the anomeric selectivity (*β*:α = 3:1) was induced when dichloromethane was employed as solvent. Attempts to further increase *β*-selectivity of this reaction by conformationally constraining the glycosyl donor through the use of a 4,6-*O*-benzylidene-protected donor proved unsuccessful. Partial hydrolysis of the cyclic acetal functionality under the acidic reaction conditions required for glycosyl phosphate activation did not allow for the use of the benzylidene constraint.[35,36]

The dramatic solvent effect prevalent in couplings with mannosyl phosphate donors was not observed when glucosamine donor **39**, containing a nonparticipating azide-masked nitrogen, was coupled to glucuronic acceptor **42** (CH_3CN: *β*:α = 1:1; CH_2Cl_2: *β*:α = 1:2.5). Likewise, no change in anomeric selectivity was detected when either acetonitrile or dichloromethane were employed in the coupling of **39** and secondary alcohol **24** (*β*:α = 1:4).

***C*-Glycosides.** Along with our efforts to prepare a range of *O*-glycosides, we were interested in exploring the utility of glycosyl phosphates in the synthesis of *C*-glycosides. Decreased susceptibility to hydrolysis in vivo renders *C*-glycosides attractive analogues to naturally occurring sugars.[37] A wide range of *C*-alkyl and *C*-aryl glycosides have been prepared and evaluated as potential pharmaceutical agents.[38] Common methods for the preparation of *C*-glycosides rely on the use of glycosyl donors such as glycosyl trichloroacetimidate, thioglycosides, glycosyl phosphites, and glycosyl fluorides.[38] Coupling of the electrophilic glycosyl donors with electron-rich aromatic systems leads to formation of *C*-aryl glycosides while *C*-alkyl glycosides are formed when silicon-based *C*-nucleophiles are utilized. Other methods for preparing *C*-glycosides involve the use of palladium-mediated couplings, sigmatropic rearrangements, carbene insertions, anomeric anions, and transition metal complexes. Despite considerable interest in this field, current technologies for the synthesis of *C*-glycosides are often hampered by the need for prolonged reaction times and poor stereoselectivity.

Previous work on *β*-*C*-aryl glucoside synthesis suggested an indirect route for anomeric−aryl bond formation via a Fries-like *O*-to-*C* rearrangement.[39,40] Upon coupling with phenolic acceptors, the initially formed *O*-glycoside rearranges to the *C*-glycoside with net retention of configuration at the anomeric center in the presence of a Lewis acid.[41] When glucosyl trichloroacetimidates[39] and fluorides[40] were used, exclusive *β*-*C*-aryl selectivity was observed. We investigated the synthesis of mannosyl and glucosyl *C*-aryl glycosides from glycosyl phosphate donors via a Fries-like rearrangement.[42] Coupling of mannosyl donor **36** with aromatic acceptors **51**−**53** furnished exclusively α-*C*-aryl glycosides **56**−**58** within 1 h at 0 °C (Table 3). Notably, a single regioisomer with regard to the aromatic system was formed when *C*-aryl glycosides **57** and **58** were synthesized. When deactivated phenols such as 3-*O*-acetyl phenol **54** served as glycosyl acceptors, isolation of the α-*O*-glycoside **59** was possible. Coupling **36** and allyltrimethylsilane afforded *C*-alkyl glycoside **60** in high yield (93%). The complete stereospecificity observed in *C*-mannoside formation with perbenzylated donor **36** stands in stark contrast to the formation of anomeric mixtures obtained for *O*-glycoside formation.

Table 3. Formation of *C*-Glycosides with Glycosyl Phosphates[a]

Donor	Acceptor	Product	Yield (%)[a]
36	 **51**	 **56**	85
36	 **52**	 **57**	79
36	 **53**	 **58**	82
36	 **54**	 **59**	73
36	 **55**	 **60**	93
 **50**	**51**	 **61**	57
		 **62**	13[b]

[a] All reactions were carried out under the following conditions unless otherwise noted: 1.2 equiv of acceptor, 1.0 equiv of donor, 1.2 equiv of TMSOTf in CH_2Cl_2 at 0 °C for 1 h. [b] When the reaction of **50** and **51** was carried out at −15 °C for 15 min, **62** was isolated in 79% yield.

(33) Flowers, H. M. *Carbohydr. Res.* **1983**, *119*, 75.
(34) Marra, A.; Esnault, J.; Veyrieres, A.; Sinay, P. *J. Am. Chem. Soc.* **1992**, *114*, 6354.
(35) (a) Crich, D.; Sun, S. *J. Org. Chem.* **1996**, *61*, 4506. (b) Weingart, R.; Schmidt, R. R. *Tetrahedron Lett.* **2000**, *41*, 8753.
(36) For the synthesis of the α-mannoside component of Bleomycin A_2 with phosphate donors see: Boger, D. L.; Honda, T. *J. Am. Chem. Soc.* **1994**, *116*, 5647.
(37) For a review see: Levy, D. E.; Tang, C. *The Chemistry of C-Glycosides*; Pergamon: New York, 1995; Vol. 13.
(38) For a review see: Postema, M. H. D. *C-Glycoside Synthesis*; CRC Press: London, UK, 1995.
(39) Mahling, J.-A.; Schmidt, R. R. *Synthesis* **1993**, 325.
(40) Matsumoto, T.; Hosoya, T.; Suzuki, K. *Synlett* **1991**, 709.
(41) Kometani, U.; Kondo, H.; Fujimori, Y. *Synthesis* **1988**, 1005.
(42) Palmacci, E. R.; Seeberger, P. H. *Org. Lett.* **2001**, *3*, 1547.

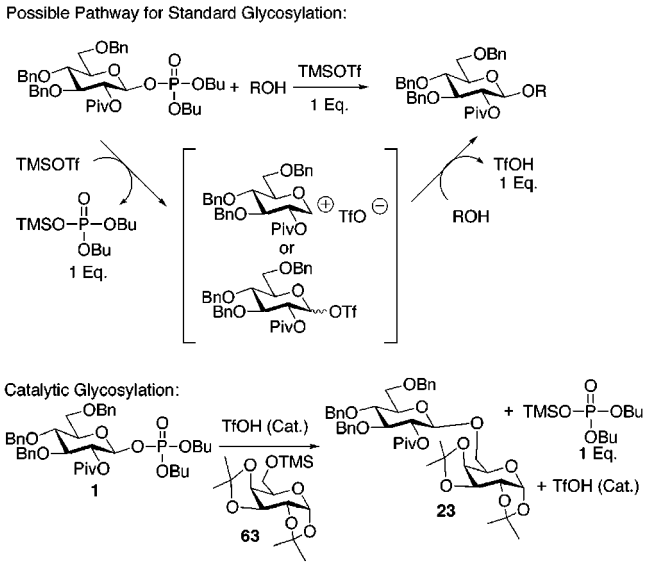


Figure 3. Analysis of TMSOTf-mediated glycosylations.

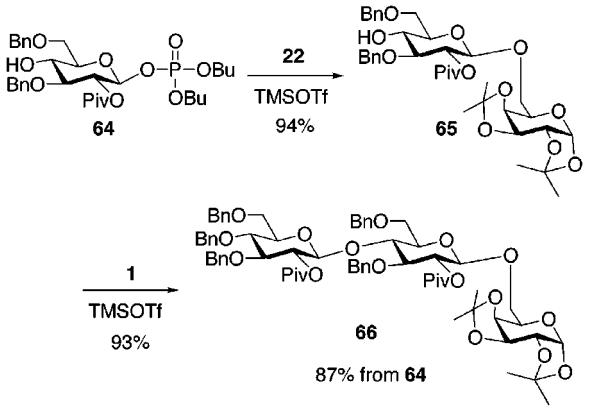
Glucosyl phosphate **50** was employed in the synthesis of *C*-aryl glycosides, however, with reduced yields. Activation of **50** with TMSOTf in the presence of 3,4,5-trimethoxyphenol **51** afforded the α-*O*-glycoside product **62** in good yield (79%) in just 15 min at 0 °C. Prolonged reaction times (2−4 h) and warming of the reaction mixture to room temperature provided the desired *β*-*C*-aryl glucoside **61** in modest 57% yield. No *β*-*O*-glycoside products were formed under any of the reaction conditions explored.

Catalytic Activation. The need for a stoichiometric amount of TMSOTf to efficiently promote the coupling of glycosyl phosphates with *O*- and *C*-nucleophiles prompted us to explore catalytic alternatives. In the screening of reagents for phosphate activation we found that protic acids (TsOH, TfOH, and CSA) were ineffective for this purpose. On the contrary, silyl triflates were excellent activators, affording effective glycosylations with a variety of donors. We propose that a driving force of glycosylations utilizing phosphate donors is the formation of a stoichiometric amount of silyl phosphate as a byproduct. The release of silyl phosphates and phosphites has been observed previously in other systems.[12,43]

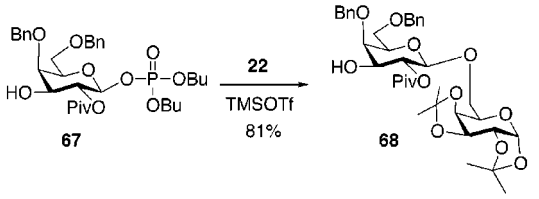
We investigated activation of phosphate donor **1** with catalytic protic acid in the presence of trimethylsilyl-protected acceptor **63** (Figure 3).[44] Varying amounts of triflic acid were added at low temperature (−78 °C) to induce glycosylation. Remarkably, as little as 1 mol % of triflic acid afforded coupled product in excellent yield (91%). This suggests that the in situ generation of catalytic amounts of trimethylsilyl triflate is sufficient for competent glycosylation. Although this analysis does not disprove other mechanistic pathways, the activation of glycosyl phosphates in a catalytic fashion may prove useful in expanding their utility in carbohydrate synthesis.[45]

Regioselective Glycosylations. The necessity of selectively removing protecting groups at each step of an oligosaccharide synthesis is tedious and time-consuming. After establishing the utility of glycosyl phosphates as glycosylating agents, we were interested in the development of a novel orthogonal method that would eliminate the need for intermediate protecting group removal. To carry out the synthesis of oligosaccharides with minimal protecting group transformations, a regioselective glycosylation strategy was envisioned. Fundamental to this approach was the use of a central building block capable of displaying both donor and acceptor properties. The central *β*-glycosyl phosphate building block **64** revealed a C4 hydroxyl group and was prepared from the corresponding 4-*O*-*tert*-butyldimethysilyl glycosyl phosphate by treatment with TBAF.[46]

Scheme 3. Regioselective Glycosylation with C4−OH Glucosyl Phosphate **64**



Scheme 4. Regioselective Glycosylation with C3−OH Galactosyl Phosphate **67**



Activation of donor **64** at −78 °C in the presence of primary alcohol **22** afforded *β*-(1→6)-linked disaccharide **65** bearing a C4-hydroxyl group in excellent yield (94%) as the only coupled product (Scheme 3). Following chromatography, subsequent glycosylation of disaccharide **65** containing a unique C4-hydroxyl with glucosyl phosphate **1** provided trisaccharide **66** in 87% overall yield in only two steps. When both glycosylations were carried out in a one-pot procedure without intermediate purification, trisaccharide **66** was isolated in 72% overall yield. This method may prove useful in the synthesis of naturally occurring glycosphingolipids[47] where a central glucosyl residue is C4-glycosylated and *β*-linked to a lipid via the anomeric position.[48]

To further explore the scope of regioselective glycosylations we prepared *β*-galactosyl phosphate **67** bearing a free hydroxyl group on C3. Activation of **67** in the presence of primary alcohol **22** afforded *β*-(1→6) linked disaccharide **68** in excellent yield (81%) (Scheme 4). Importantly, no other coupled products were observed under these reaction conditions.

After establishing the regioselective glycosylation of donors exposing C3 and C4 hydroxyl groups, we examined the use of

(43) Schmidt, R. R. *Carbohydrates-Synthetic Methods and Application in Medicinal Chemistry*; Ogura, H., Hasegawa, A., Suami, T., Eds.; VCH Verlagsgesellschaaft mbH: Weinheim, Germany, 1992; pp 66−88.

(44) For glycosylations with TMS-OR acceptors see: (a) Mukaiyama, T.; Matsubara, K. *Chem. Lett.* **1992**, 1041 and references therein. (b) Nashed, E. M.; Glaudemans, C. P. J. *J. Org. Chem.* **1989**, *54*, 6116.

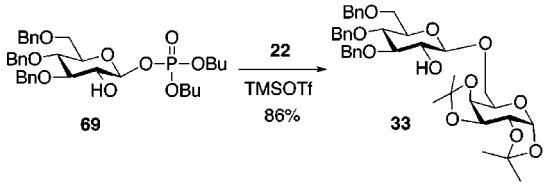
(45) For the benzylation of substrates containing benzylidene functionality with 0.1 equiv of triflic acid as an activator see: Wessel, H. P.; Iversen, T.; Bundle, D. R. *J. Chem. Soc., Perkin Trans. 1* **1985**, 2247.

(46) Andrade, R. B.; Plante, O. J.; Melean, L. G.; Seeberger, P. H. *Org. Lett.* **1999**, *1*, 1811.

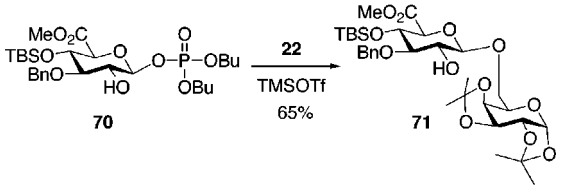
(47) For previous syntheses of glycosphingolipids see: (a) Schmidt, R. R.; Zimmermann, P. *Angew. Chem.* **1986**, *98*, 722. (b) Prabhanjan, H.; Ishida, H.; Kiso, M.; Hasegawa, A. *Carbohydr. Res.* **1991**, *211*, C1−C5.

(48) Varki, A. *Essentials of Glycobiology*; Varki, A., Cummings, R., Esko, J., Freeze, H., Hart, G., Marth, J., Eds.; Cold Spring Harbor Press: Cold Spring Harbor, NY, 1999; pp 115−129.

Scheme 5. Regioselective Glycosylation with C2−OH Glucosyl Phosphate **69**



Scheme 6. Regioselective Glycosylation with C2−OH Glucuronic Acid Phosphate **70**



donors in which the C2 position was unprotected. On the basis of prior observations with donor **9**, we anticipated that any productive coupling events should afford β-linked compounds even without the use of a participating group on C2.[18a] β-Glycosyl phosphate **69**[16g] was prepared and coupled with primary alcohol **22** (Scheme 5). After 10 min at −78 °C, 2-OH disaccharide **33** (86%) was formed as the only product. The exclusive β-selectivity observed in this example is in accordance with earlier reports by Ikegami that described similar stereoselective glycosylations when benzyl ethers were installed on C2 of phosphate donors.[18a] These examples demonstrate that *trans*-glycosidic linkages are readily formed with glycosyl phosphates whereas *cis*-glycosidic linkages are potentially a limitation.

Another example of regioselective glycosylations with C2-OH donor **70** (Scheme 6) also proceeded with complete β-selectivity. Glucuronic acid phosphate **70** was prepared and coupled with **22**. The reaction was significantly more rapid than with donor **19** bearing a C2-ester and afforded disaccharide **71** as the sole coupled product in good yield (65%). The reactivity of **70** as a glycosyl donor is important in light of long reaction times required for most uronic acid donors.

Glycosyl Dithiophosphates. In addition to glycosyl phosphates, we were interested in exploring the synthesis and use of phosphorus analogues as glycosylating agents. A number of phosphate analogues including dimethylphosphinothioates,[49] phosphorimidates,[50] and phosphoramidates[51] had been previously applied to carbohydrate chemistry. We chose to synthesize and evaluate glycosyl dithiophosphate triesters[52] in which the bridging and phosphoryl oxygens are replaced by sulfur atoms. Our choice was based on reasoning that these sites of modification would most greatly influence the stability and reactivity of the donor.

Glycosyl dithiophosphates were prepared in a fashion analogous to that described for glycosyl phosphates. Epoxidation of the glycal with DMDO afforded the 1,2-anhydrosugar that was opened with commercially available *O*,*O*-diethyldithiophosphate

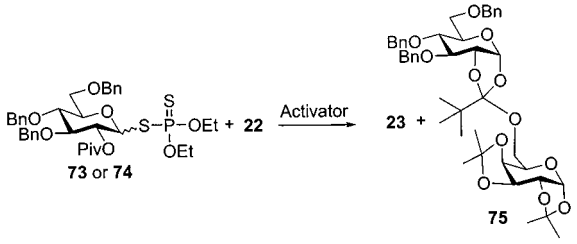
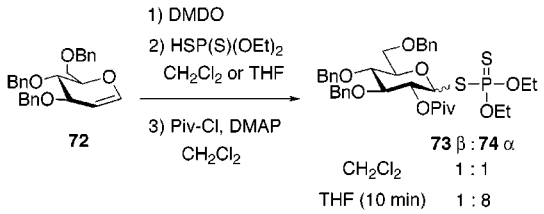


Figure 4. Reagents screened for dithiophosphate activation.

Scheme 7. Synthesis of Glycosyl Dithiophosphates



to afford the C2-OH glycosyl dithiophosphate in good yield (82−88%).[19] The opening with *O*,*O*-diethyldithiophosphate also exhibited a strong solvent dependence regarding the formation of different anomers (CH_2Cl_2: β:α = 1:1; THF: β:α = 1:8) (Scheme 7). Unlike the corresponding glycosyl phosphates, C2-OH glycosyl dithiophosphates were found to be completely stable to silica column chromatography. However, as was the case with glycosyl phosphates, treatment of C2-OH glycosyl dithiophosphates with NaH and benzyl bromide resulted in the formation of C2-thiophosphoryl benzyl glycosides. Acylation of C2-OH glycosyl dithiophosphates successfully furnished fully protected donors **73** and **74**. A major attribute of this method is the efficiency of preparation of glycosyl dithiophosphates (82−88%) when compared to the lower yields (55−75%) reported for the preparation of thioglycosides or *n*-pentenyl glycosides from glycals.[53]

Prior work with 2-deoxy dithiophosphates suggested that thiophilic reagents such as silver salts (AgOTf, $AgClO_4$) and iodonium sources ($I(coll)_2ClO_4$, *N*-iodosuccinimide) may serve as activators for glucosyl dithiophosphate donors.[54] Unexpectedly, low yields (5−38%) were obtained upon reaction of **73** or **74** and **22** in the presence of various promoters (Figure 4). Activation with *N*-iodosuccinimide for 16 h resulted in the formation of the desired β-linked disaccharide **23** (11%) and ortho ester **75** (27%). This finding was unusual considering that the pivaloyl group is widely used to prevent ortho ester formation during glycosylation.

In the search for more efficient promoters of β-selective glycosylations, we explored coupling conditions commonly used for thioglycoside donors (Figure 4). The coupling of either α- or β-glycosyl dithiophosphates with **22** using excess methyl triflate (MeOTf) as an activator in the presence of molecular sieves and 2,6-di-*tert*-butylpyridine (DTBP) proceeded in modest yield (70%). Contrary to results with glycosyl phosphates, no reactivity difference for glycosyl dithiophosphate anomers was found. Hindered secondary acceptors and substrates containing acid-sensitive glycals were glycosylated under MeOTf/DTBP conditions and resulted in coupling yields comparable to those obtained when thioglycosides are subjected to the same conditions.[19]

Application of another thiophilic activator, dimethylthiomethylsulfonium triflate (DMTST),[55] to the coupling of dithio-

(49) Yamanoi, T.; Nakamura, K.; Sada, S.; Goto, M.; Furusawa, Y.; Takano, M.; Fujioka, A.; Yanagihara, K.; Satoh, Y.; Hosokawa, H.; Inazu, T. *Bull. Chem. Soc. Jpn.* **1993**, *66*, 2617.

(50) Pan, S.; Li, H.; Hong, F.; Yu, B.; Zhao, K. *Tetrahedron Lett.* **1997**, *38*, 6139.

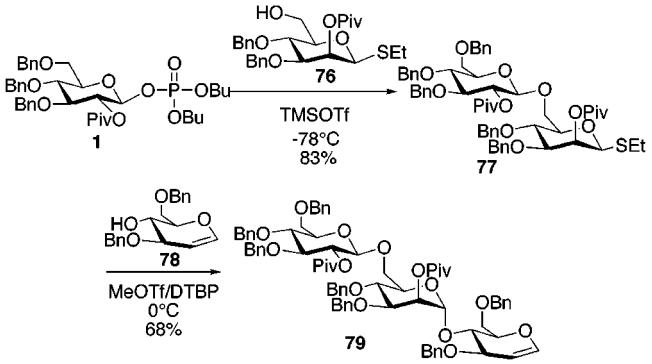
(51) (a) Hashimoto, S.-I.; Sakamoto, H.; Honda, T.; Ikegami, S. *Tetrahedron Lett.* **1997**, *38*, 5181. (b) Hashimoto, S.-I.; Sakamoto, H.; Honda, T.; Abe, H.; Nakamura, S.-I.; Ikegami, S. *Tetrahedron Lett.* **1997**, *38*, 8969. (c) Chen, M.-J.; Ravindran, K.; Landry, D. W.; Zhao, K. *Heterocycles* **1997**, *45*, 1247.

(52) Referred to as glycosyl dithiophosphates in the remaining text.

(53) Seeberger, P. H.; Eckhardt, M.; Gutteridge, C. E.; Danishefsky, S. J. *J. Am. Chem. Soc.* **1997**, *119*, 10064.

(54) (a) Bielawska, H.; Michalska, M. *J. Carbohydr. Chem.* **1991**, *10*, 107. (b) Laupichler, L.; Sajus, H.; Thiem, J. *Synthesis* **1992**, 1133.

Scheme 8. Orthogonal Glycosylations with Glycosyl Phosphates and Thioglycosides



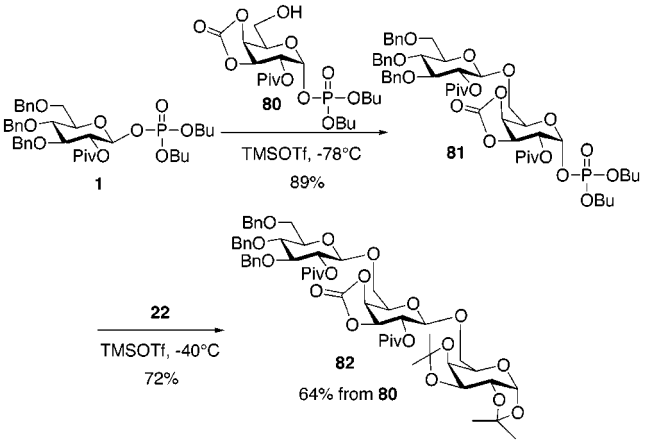
phosphate donor **73** and **22** resulted in the formation of **23** in excellent yield (94%) (Figure 4). The use of DMTST as an activator for glycosyl dithiophosphate donors, in conjunction with their ease of synthesis, should lead to a more widespread use of this class of donors for the synthesis of oligosaccharides.

Orthogonal Glycosylation (Phosphate/Thiodonor). A number of orthogonal glycosylation strategies have been developed for the synthesis of oligosaccharides both in solution and on a solid support.[56] On the basis of our findings that ethanethiol was a suitable acceptor in TMSOTf-mediated glycosyl phosphate glycosylations (Table 1), we envisioned an orthogonal glycosylation strategy employing glycosyl phosphates and thiodonors. We designed a synthesis whereby activation of a phosphate donor with TMSOTf in the presence of a thioglycoside acceptor would lead to formation of a disaccharide thioglycoside. Further elongation of the disaccharide could be accomplished by thioglycoside activation.

In reducing this principle to practice, thioglycoside **76** bearing a free hydroxyl group was selected to function as a central building block (Scheme 8). Activation of **1** with TMSOTf at −78 °C in the presence of **76** furnished disaccharide **77** in good yield. Coupling of **77** with glycal acceptor **78** was affected by MeOTf/DTBP activation to afford trisaccharide **79**. The orthogonality of glycosyl phosphates and thioglycosides provides a convenient two-step method of preparing trisaccharides from a central building block.

Anomer-Controlled Glycosylation. The regioselective and orthogonal glycosylation strategies detailed above had previously been established for glycosyl donors other than glycosyl phosphates. Control of donor reactivity via the anomeric configuration is a strategy that to our knowledge has not previously been explored. As outlined above, we developed conditions that allowed for the synthesis of either α- or *β*-anomers of glycosyl phosphates in excellent yield. Importantly, the more reactive *β*-glycosyl phosphates in the glucose and galactose series were activated at −78 °C while their α-anomeric counterparts were inert under those conditions and required higher temperatures (−40 → −20 °C) for activation. Here we describe an orthogonal glycosylation strategy that takes advantage of the reactivity differences of α- and *β*-glycosyl phosphates.

Scheme 9. Orthogonal Glycosylations Based on Anomeric Configuration



α-Galactosyl phosphate **80** exposing a 6-hydroxyl group was chosen as the central building block for the synthesis of a trisaccharide (Scheme 9). Monomer **80** was prepared from 6-*O*-triisopropylsilyl-3,4-*O*-carbonyl galactal in four steps and 62% overall yield.[57,58] Glycosylation of **80** with *β*-glucosyl phosphate **1** at −78 °C resulted exclusively in *β*-phosphate activation and afforded as anticipated *β*-(1→6)-linked disaccharide α-phosphate **81**. Following chromatography, disaccharide **81** was coupled with **22** at −40 °C to afford trisaccharide **82** in two steps and good overall yield (64%). Anomer-controlled glycosylations with α- and *β*-phosphates provide a novel alternative to existing methods for the synthesis of oligosaccharides.

Summary

In conclusion, we have developed an efficient one-pot synthesis of α- and *β*-glycosyl phosphate and dithiophosphate triesters from glycals via 1,2-anhydrosugars. The resulting glycosyl phosphates have been shown to function as powerful glycosylating agents for the installation of *β*-glucosidic, *β*-galactosidic, α-fucosidic, α-mannosidic, *β*-glucuronic acid, and *β*-glucosamine linkages even with hindered and electron-deficient substrates. In addition to serving as efficient donors for *O*-glycosylations, glycosyl phosphates are effective in the preparation of *S*-glycosides and *C*-glycosides. Not only can glycosyl phosphates be activated by stoichiometric amounts of TMSOTf but the acid-catalyzed coupling of glycosyl phosphates with silylated acceptors is also discussed. Glycosyl dithiophosphates are activated with different thiophiles under basic conditions that are compatible with acceptors containing acid-sensitive functional groups.

To minimize protecting group manipulations, orthogonal and regioselective glycosylation strategies with glycosyl phosphates are reported. An orthogonal glycosylation method involving the activation of a glycosyl phosphate donor in the presence of a thioglycoside acceptor is detailed. Also, an acceptor-mediated regioselective glycosylation strategy is described. Finally, a unique glycosylation strategy exploiting the difference in reactivity of α- and *β*-glycosyl phosphates is disclosed. The protocols developed here demonstrate the versatility of glycosyl phosphates and glycosyl dithiophosphates for the construction of glycosidic linkages. Extension of this work to the automated

(55) (a) Fugedi, P.; Garegg, P. J. *Carbohydr. Res.* **1986**, *149*, 9. (b) Wong, C.-H.; Ye, X.-S.; Zhang, Z. *J. Am. Chem. Soc.* **1998**, *120*, 7137.

(56) (a) Fraser-Reid, B.; Udodong, U. E.; Wu, Z.; Ottosson, H.; Merritt, J. R.; Rao, C. S.; Roberts, C. *Synlett* **1992**, 927. (b) Raghavan, S.; Kahne, D. *J. Am. Chem. Soc.* **1993**, *115*, 1580. (c) Geurtsen, R.; Holmes, D. S.; Boons, G.-J. *J. Org. Chem.* **1997**, *62*, 8145. (d) Douglas, N. L.; Ley, S. V.; Lucking, U.; Warriner, S. L. *J. Chem. Soc., Perkin Trans. 1* **1998**, 51. (e) Zhu, T.; Boons, G.-J. *Angew. Chem., Int. Ed. Engl.* **1998**, *37*, 1898. (f) Zhang, Z.; Ollmann, I. R.; Ye, X.-S.; Wischnat, R.; Baasov, T.; Wong, C.-H. *J. Am. Chem. Soc.* **1999**, *121*, 734.

(57) (1) DMDO; (2) $HOP(O)(OBu)_2$, THF; (3) Piv-Cl, DMAP; (4) TBAF, AcOH, room temperature, 15 min (62% overall).

(58) For the synthesis of 6-*O*-TIPS-3,4-di-*O*-carbonyl galactal see: Gervay, J.; Peterson, J. M.; Oriyama, T.; Danishefsky, S. J. *J. Org. Chem.* **1993**, *58*, 5465.

solid-phase synthesis of oligosaccharides and glycoconjugates is currently underway in our laboratory.

Experimental Section

Synthesis of β-Glycosyl Phosphates: General Procedure A. Suitably protected glycal (1.0 equiv) was dissolved in CH_2Cl_2 (1 mL/0.10 mmol glycal) and cooled to 0 °C. A 0.08 M solution of dimethyldioxirane in acetone (1.2 equiv) was added and the reaction was stirred for 15 min. After the solvent was removed in vacuo, the residue was dissolved in CH_2Cl_2 (2 mL/0.10 mmol glycal). The solution was cooled to −78 °C for 15 min. A solution of dialkyl phosphate (1.1 equiv) in CH_2Cl_2 (2 mL/0.10 mmol glycal) was added dropwise over 5 min. After addition was complete, the reaction mixture was warmed to 0 °C and DMAP (4 equiv) and pivaloyl chloride (2 equiv) were added. The solution was warmed to room temperature over 1 h. The addition of 40% EtOAc/hexanes afforded a white precipitate that was filtered off through a pad of silica. The eluent was concentrated and purified by flash silica column chromatography (short plug) to afford β-enriched glycosyl phosphates.

Synthesis of α-Glycosyl Phosphates: General Procedure B. Suitably protected glycal (1.0 equiv) was dissolved in CH_2Cl_2 (1 mL/0.10 mmol glycal) and cooled to 0 °C. A 0.08 M solution of dimethyldioxirane in acetone (1.2 equiv) was added and the reaction was stirred for 15 min. After the solvent was removed in vacuo, the residue was redissolved in THF (2 mL/0.10 mmol glycal). The solution was cooled to −78 °C for 15 min. A solution of dialkyl phosphate (1.1 equiv) in THF (2 mL/0.10 mmol glycal) was added dropwise over 5 min. After addition was complete, the reaction mixture was warmed to 0 °C and DMAP (4 equiv) and pivaloyl chloride (2 equiv) were added. The solution was warmed to room temperature over 1 h. The addition of 40% EtOAc/hexanes afforded a white precipitate that was filtered off through a pad of silica. The eluent was concentrated and purified by flash silica column chromatography (short plug) to afford α-enriched glycosyl phosphates.

Synthesis of 2-*O*-Triethylsilyl Glycosyl Phosphates: General Procedure C. Suitably protected glycal (1.0 equiv) was dissolved in CH_2Cl_2 (1 mL/0.10 mmol glycal) and cooled to 0 °C. A 0.08 M solution of dimethyldioxirane in acetone (1.2 equiv) was added and the reaction was stirred for 15 min. After the solvent was removed in vacuo, the residue was redissolved in THF (2 mL/0.10 mmol glycal). The solution was cooled to −78 °C for 15 min. A solution of dialkyl phosphate (1.1 equiv) in THF (2 mL/0.10 mmol glycal) was added dropwise over 5 min. After addition was complete, the reaction mixture was warmed to room temperature and imidazole (3.5 equiv) and triethylsilyl chloride (2.5 equiv) were added. After 2 h at room temperature, the reaction mixture was diluted with EtOAc (50 mL) and washed with saturated $NaHCO_3$(aq), brine, and water. After extraction of the aqueous layers with 2 × 50 mL of EtOAc, the organics were dried over Na_2SO_4, filtered, and concentrated. Purification by flash silica column chromatography afforded 2-*O*-triethylsilylglycosyl phosphates.

Glycosyl Phosphate Couplings: General Procedure D. Glycosyl phosphate donor (1.2 equiv) and acceptor (1.0 equiv) were combined and azeotropically dried with toluene (3 × 5 mL) followed by 1 h under vacuum. The mixture was dissolved in CH_2Cl_2 (2 mL/0.10 mmol acceptor) and cooled to −78 °C for 15 min before trimethylsilyltriflate (1.2 equiv) was added dropwise. After the mixture was stirred for 30 min at −78 °C, triethylamine (2 equiv) was added. The solution was warmed to room temperature and the solvent was removed in a stream of N_2. The resulting mixture was purified by flash silica column chromatography.

Dibutyl 2-*O*-Pivaloyl-3,4,6-tri-*O*-benzyl-β-D-glucopyranoside Phosphate 1. General procedure A with 1,5-anhydro-2-deoxy-3,4,6-tri-*O*-benzyl-D-*arabino*-hex-1-enitol (1.00 g, 2.41 mmol), dimethyldioxirane (36.0 mL, 2.90 mmol), dibutyl phosphate (0.50 mL, 2.5 mmol), pivaloyl chloride (0.59 mL, 4.8 mmol), and DMAP (1.18 g, 9.64 mmol) afforded 1.69 g (91%, 11:1 β:α) of **1** as a colorless oil after flash silica column chromatography (40→50% EtOAc/hexanes). $[\alpha]^{24}_D$ −1.9° (*c* 1.50, CH_2Cl_2); IR (thin film) 2946, 1740, 1454, 1282, 1016 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 7.33−7.25 (m, 13H), 7.16−7.14 (m, 2H), 5.24 (app t, *J* = 7.3 Hz, 1H), 5.17 (app t, *J* = 8.5 Hz, 1H), 4.80−4.75 (m, 2H), 4.70 (d, *J* = 11.0 Hz, 1H), 4.69−4.54 (m, 2H), 4.51 (d, *J* = 11.0 Hz, 1H), 4.08−4.00 (m, 4H), 3.82 (t, *J* = 9.5 Hz, 1H), 3.78−3.70 (m, 3H), 3.64−3.61 (m, 1H), 1.64−1.59 (m, 4H), 1.40−1.34 (m, 4H), 1.20 (s, 9H), 0.96−0.88 (m, 6H);^{13}C NMR (125 MHz, $CDCl_3$) δ 177.2, 138.2, 138.1, 128.7, 128.3, 128.2, 128.1, 128.0, 127.6, 97.0 (d, J_{C-P} = 5.0 Hz), 83.1, 76.2, 75.9, 73.9, 73.3, 68.4, 68.2, 68.1, 39.2, 32.7, 26.9, 19.1, 14.0; ^{31}P NMR (200 MHz, $CDCl_3$) δ -2.2; FAB MS *m*/*z* $(M)^+$ calcd 726.3532, obsd 726.3537.

Dibutyl 2-*O*-Pivaloyl-3,4,6-tri-*O*-benzyl-α-D-glucopyranoside Phosphate 2. General procedure B with 1,5-anhydro-2-deoxy-3,4,6-tri-*O*-benzyl-D-*arabino*-hex-1-enitol (0.192 g, 0.462 mmol), dimethyldioxirane (8.7 mL, 0.69 mmol), dibutyl phosphate (0.100 mL, 0.508 mmol), pivaloyl chloride (85.0 μL, 693 μmol), and DMAP (0.169 g, 1.39 mmol) afforded 0.169 g (59%, 1:4 β:α) of **2** as a colorless oil after flash silica column chromatography (40→50% EtOAc/hexanes). $[\alpha]^{24}_D$ +50.5° (*c* 0.63, CH_2Cl_2); IR (thin film) 2960, 2872, 1736, 1454, 1282 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 7.35−7.27 (m, 13 H), 7.18−7.15 (m, 2H), 5.85 (dd, *J* = 1.8, 6.4 Hz, 1H), 4.99−4.97 (m, 1H), 4.83−4.80 (m, 3H), 4.63 (d, *J* = 11.5 Hz, 1H), 4.56−4.50 (m, 3H), 4.10−4.02 (m, 5H), 3.86−3.79 (m, 2H), 3.68 (d, *J* = 11.0 Hz, 1H), 1.86−1.61 (m, 4H), 1.44−1.36 (m, 4H), 1.24 (s, 9H), 0.97−0.91 (m, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 177.7, 138.3, 138.1, 138.0, 128.6, 128.5, 128.3, 128.2, 128.1, 128.0, 127.9, 127.7, 127.6, 94.7 (d, J_{C-P} = 5.5 Hz), 79.5, 75.6, 75.4, 73.7, 72.7, 72.6, 68.2, 68.0, 67.9, 67.8, 39.0, 32.5, 32.4, 27.3, 18.8, 13.8; ^{31}P NMR (200 MHz, $CDCl_3$) δ −2.5; FAB MS *m*/*z* $(M)^+$ calcd 726.3532, obsd 726.3537.

Dibutyl 3,4,6-Tri-*O*-benzyl-2-*O*-triethylsilyl-D-glucopyranoside Phosphates 9 and 10. General procedure C with 1,5-anhydro-2-deoxy-3,4,6-tri-*O*-benzyl-D-*arabino*-hex-1-enitol (0.295 g, 0.495 mmol), dimethyldioxirane (9.0 mL, 0.70 mmol), dibutyl phosphate (0.108 mL, 0.545 mmol), imidazole (50.0 mg, 0.740 mmol), and triethylsilyl chloride (0.10 mL, 0.59 mmol) afforded 359 mg (79%, 2:1, β:α) of **9** and **10** as colorless oils after flash silica column chromatography (30→40%EtOAc/hexanes).

Dibutyl 3,4,6-tri-*O*-benzyl-2-*O*-triethylsilyl-β-D-glucopyranoside phosphate 9: $[\alpha]^{24}_D$ −8.3° (c 4.39, CH_2Cl_2); IR (thin film) 2976, 2870, 1460, 1130 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 7.37−7.25 (m, 13H), 7.12−7.09 (m, 2H), 5.02 (dd, *J* = 6.0, 7.5 Hz, 1H), 4.93−4.86 (m, 2H), 4.75 (d, *J* = 11.0 Hz, 1H), 4.61−4.50 (m, 2H), 4.13−4.08 (m, 3H), 3.75−3.67 (m, 4H), 3.61−3.58 (m, 1H), 3.55 (app t, *J* = 8.6 Hz, 1H), 1.69−1.60 (m, 4H), 1.45−1.38 (m, 4H), 1.00−0.89 (m, 15H), 0.68 (app q, *J* = 8.0 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 139.0, 138.3, 138.2, 128.7, 128.6, 128.2, 128.1, 128.0, 127.6, 127.5, 99.5 (d, J_{C-P} = 6.4 Hz), 85.8, 77.9, 75.8, 75.6, 75.5, 75.4, 75.2, 73.8, 68.8, 68.0, 67.9, 32.6, 32.5, 19.0, 14.0, 13.9, 7.2, 5.3; ^{31}P NMR (200 MHz, $CDCl_3$) δ −1.4; FAB MS *m*/*z* $(M)^+$ calcd 756.3822, obsd 756.3822.

Dibutyl 3,4,6-tri-*O*-benzyl-2-*O*-triethylsilyl-α-D-glucopyranoside phosphate 10: $[\alpha]^{24}_D$ +44.1° (*c* 1.50, CH_2Cl_2); IR (thin film) 2976, 2870, 1460, 1130 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 7.37−7.26 (m, 13H), 7.10−7.09 (m, 2H), 5.65 (dd, *J* = 2.5, 6.3 Hz, 1H), 4.93 (d, *J* = 11.5 Hz, 1H), 4.83−4.79 (m, 2H), 4.62 (d, *J* = 12.0 Hz, 1H), 4.51−4.47 (m, 2H), 4.12−3.98 (m, 5H), 3.84−3.70 (m, 4H), 3.65 (d, *J* = 10.0 Hz, 1H), 1.70−1.60 (m, 4H), 1.45−1.34 (m, 4H), 1.02−0.89 (m, 15H), 0.67 (app q, *J* = 8.0 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 138.9, 138.3, 138.0, 128.6, 128.5, 128.2, 128.0, 127.9, 127.7, 127.6, 97.9 (d, J_{C-P} = 8.1 Hz), 82.2, 75.8, 75.3, 73.8, 73.4, 73.3, 72.5, 68.3, 67.8, 67.6, 67.5, 32.5, 32.4, 18.9, 18.8, 13.8, 7.0, 5.1; ^{31}P NMR (200 MHz, $CDCl_3$) δ −2.3; FAB MS *m*/*z* $(M)^+$ calcd 756.3822, obsd 756.3823.

3,4,6-Tri-*O*-benzyl-2-*O*-pivaloyl-β-D-glucopyranosyl-(1→6)-1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranoside 23. General procedure D with donor **1** (57.5 mg, 77.0 μmol), acceptor **22** (13.3 mg, 51.0 μmol), and TMSOTf (14.0 μL, 77.0 μmol) afforded 37.2 mg (94%) of **23** as a colorless oil after flash silica column chromatography (25% EtOAc/hexanes). $[\alpha]^{24}_D$ −45.2° (*c* 2.34, CH_2Cl_2); IR (thin film) 3029, 2978, 2933, 2904, 1741, 1134, 1028 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 7.39−7.24 (m, 13H), 7.19−7.15 (m, 2H), 5.49 (d, *J* = 5.0 Hz, 1H), 5.10 (app t, *J* = 8.5 Hz, 1H), 4.79−4.69 (m, 3H), 4.64 (d, *J* = 8.0 Hz, 1H), 4.58−4.53 (m, 3H), 4.46 (d, *J* = 8.0 Hz, 1H), 4.29−4.25 (m, 2H), 4.10−4.07 (m, 1H), 3.97−3.94 (m, 1H), 3.76−3.69 (m, 4H), 3.63−3.59 (m, 1H), 3.53−3.50 (m, 1H), 1.51 (s, 3H), 1.43 (s, 3H), 1.32 (s,

3H), 1.31 (s, 3H), 1.21 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 177.1, 138.5, 138.4, 128.7, 128.6, 128.2, 128.1, 128.0, 127.9, 127.8, 127.7, 109.5, 108.8, 101.8, 96.6, 83.6, 78.1, 75.7, 75.2, 73.9, 73.3, 71.5, 70.9, 69.0, 67.4, 39.1, 27.5, 26.4, 26.3, 25.4, 24.7; FAB MS *m*/*z* $(M)^+$ calcd 776.3772, found 776.3770.

***n*-Pentenyl 2-*O*-Benzoyl-3,4,6-tri-*O*-benzyl-*β*-D-galactopyranosyl-(1→4)-3-*O*-acetyl-6-*O*-benzyl-2-deoxy-2-benzyloxycarbonylamino-*β*-D-glucopyranoside 32.** General procedure D with donor **7** (63.4 mg, 84.0 μmol), acceptor **27** (24.2 mg, 49.1 μmol), and TBSOTf (20.9 μL, 84.0 μmol) at −50 °C followed by 30 min at −20 °C afforded 50.0 mg (96%) of **32** as a colorless oil after flash silica column chromatography (30%→50% EtOAc/hexanes). $[\alpha]^{24}_D$ +29.3° (*c* 1.10, CH_2Cl_2); IR (thin film) 3029, 2872, 1726, 1540, 1453, 1367, 736; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.05 (d, J = 7.0 Hz, 1H), 7.70 (t, J = 7.5 Hz, 1H), 7.57−7.27 (m, 28H), 5.87−5.85 (m, 1H), 5.62 (dd, J = 8.0, 10.0 Hz, 1H), 5.22 (s, 2H), 5.11 (d, J = 11.5 Hz, 1H), 5.10−5.04 (m, 2H), 4.92−4.90 (m, 1H), 4.74 (d, J = 12.5 Hz, 1H), 4.70 (d, J = 12.0 Hz, 1H), 4.63 (d, J = 12.5 Hz, 1H), 4.57 (s, 2H), 4.56 (d, J = 11.5 Hz, 1H), 4.38 (d, J = 12.0 Hz, 1H), 4.33 (d, J = 7.5 Hz, 1H), 4.13 (d, J = 2.5 Hz, 1H), 4.00 (app t, J = 9.0 Hz, 1H), 3.91 (dt, J = 3.0, 6.5 Hz, 1H), 3.84−3.77 (m, 3H), 3.71−3.59 (m, 6H), 3.46−3.44 (m, 2H), 2.22−2.12 (m, 2H), 1.98 (s, 3H), 1.77−1.66 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.9, 165.0, 156.1, 138.7, 138.4, 138.2, 137.8, 136.7, 133.2, 130.1, 130.0, 128.6, 128.5, 128.4, 128.1, 127.8, 127.7, 127.6, 115.0, 102.0, 100.8, 79.8, 74.7, 74.6, 73.7, 73.4, 73.1, 72.4, 72.3, 71.5, 69.1, 68.1, 66.8, 55.9, 30.1, 28.7, 20.9; FAB MS *m*/*z* $(M)^+$ calcd 1049.4561, obsd 1049.4528.

2-(2′,3′,4′,6′-Tetra-*O*-benzyl-α-D-mannopyranosyl)-3,4,5-trimethoxyphen-1-ol 56. Diphenyl 2,3,4,6-tetra-*O*-benzyl-α-D-mannopyranosyl phosphate **36** (35 mg, 45 μmol) was coevaporated with toluene and dissolved in CH_2Cl_2 (2.0 mL), and the solution was cooled to 0 °C. 3,4,5-Trimethoxyphenol **51** (25.0 mg, 136 μmol) was added, followed by the addition of TMSOTf (10.0 μL, 55.0 μmol). The reaction mixture was allowed to warm to ambient temperature over 1 h. Triethylamine (Et_3N, 15 μL) was added and the solvent was removed in vacuo. Purification by flash silica column chromatography (5:1 hexanes:EtOAc) afforded 27.0 mg (85%) of **56** as a colorless oil. $[\alpha]^{24}_D$ +12.7° (*c* 1.82, CH_2Cl_2); IR (thin film) 3364, 2933, 1621, 1495, 1362, 1100 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.40 (s, 1H), 7.38−7.26 (m, 13H), 7.21−7.10 (m, 6H), 6.27 (s, 1H), 4.92 (d, J = 11.0 Hz, 1H), 4.82 (d, J = 0.9 Hz, 1H), 4.70 (app s, 2H), 4.64 (d, J = 12.2 Hz, 1H), 4.62 (d, J = 12.0 Hz, 1H), 4.57 (d, J = 11.0 Hz, 1H), 4.54−4.50 (m, 2H), 4.17 (app t, J = 9.8 Hz, 1H), 3.90 (app d, J = 1.9 Hz, 1H), 3.82 (s, 3H), 3.79−3.71 (m, 6H), 3.70 (s, 3H), 3.57−3.54 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 153.9, 153.7, 150.2, 138.6, 138.5, 138.4, 138.2, 134.6, 128.8, 128.6, 128.6, 128.5, 128.5, 128.2, 128.2, 127.9, 127.9, 127.8, 127.8, 127.7, 127.6, 107.6, 97.0, 84.2, 79.7, 77.0, 76.8, 75.5, 74.4, 73.6, 72.4, 68.8, 61.1, 60.9, 56.0. FAB MS *m*/*z* $(M + Na)^+$ calcd 729.3010 obsd 729.3034.

3,6-Di-*O*-benzyl-2-*O*-pivaloyl-*β*-D-glucopyranosyl-(1*β*6)-1,2:3,4-di-*O*-isopropylidene-→-D-galactopyranoside 65. General procedure D with donor **64** (63.7 mg, 100. μmol), acceptor **22** (31.2 mg, 0.120 mmol), and TMSOTf (12.0 μL, 0.100 mmol) afforded 64.6 mg (94%) of **65** as a colorless oil after flash silica column chromatography (30% EtOAc/hexanes). $[\alpha]^{24}_D$ −37.2° (*c* 5.11, CH_2Cl_2); IR (thin film) 3507, 2978, 1740, 1318, 1071 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.38−7.27 (m, 10H), 5.49 (d, J = 4.9 Hz, 1H), 5.06 (dd, J = 8.2, 9.8 Hz, 1H), 4.74 (d, J = 11.3 Hz, 1H), 4.70 (d, J = 11.3 Hz, 1H), 4.62 (d, J = 11.9 Hz, 1H), 4.59−4.56 (m, 2H), 4.48 (d, J = 7.9 Hz, 1H), 4.29 (dd, J = 2.4, 4.9 Hz, 1H), 4.24 (dd, J = 1.8, 8.2 Hz, 1H), 4.06 (dd, J = 5.2, 10.4 Hz, 1H), 3.95−3.92 (m, 1H), 3.77−3.73 (m, 4H), 3.62−3.55 (m, 2H), 3.51−3.49 (m, 1H), 1.51 (s, 3H), 1.44 (s, 3H), 1.33 (s, 3H), 1.32 (s, 3H), 1.23 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 177.0, 138.4, 137.9, 128.6, 128.0, 127.9, 127.7, 109.3, 108.7, 101.7, 96.4, 82.8, 74.5, 74.4, 73.9, 72.6, 72.0, 71.3, 70.7, 70.2, 68.9, 67.2, 38.9, 27.3, 26.3, 26.1, 25.2, 24.5; ^{31}P NMR (120 MHz, $CDCl_3$) δ −3.3; ESI MS *m*/*z* $(M + Na)^+$ calcd 709.3194, obsd 709.3161.

4,6-Di-*O*-benzyl-2-*O*-pivaloyl-*β*-D-galactopyranosyl-(1→6)-1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranoside 68. General procedure D with donor **67** (40.8 mg, 64.0 μmol), acceptor **22** (18.3 mg, 70.4 μmol), and TMSOTf (11.8 μL, 64.0 μmol) at −78 °C for 10 min followed by 30 min at −0 °C afforded 38.3 mg (79%) of **68** as a colorless oil after flash silica column chromatography (35% EtOAc/hexanes). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.39−7.27 (m, 10H), 5.48 (d, J = 5.0 Hz, 1H), 4.97 (dd, J = 8.0, 9.9 Hz, 1H), 4.72−4.69 (m, 2H), 4.58−4.47 (m, 3H), 4.45 (d, J = 8.0 Hz, 1H), 4.27 (dd, J = 2.5, 5.0 Hz, 1H), 4.21 (dd, J = 1.7, 8.0 Hz, 1H), 4.03 (dd, J = 4.7, 10.4 Hz, 1H), 3.94−3.89 (m, 2H), 3.70−3.58 (m, 5H), 2.40 (d, J = 9.6 Hz, 1H), 1.48 (s, 3H), 1.43 (s, 3H), 1.31 (app s, 6H), 1.23 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 179.2, 138.2, 137.8, 128.7, 128.7, 128.3, 128.1, 109.3, 108.7, 101.5, 96.4, 76.6, 75.6, 73.7, 73.5, 73.4, 73.4, 71.3, 70.7, 70.6, 69.0, 68.2, 67.2, 39.1, 27.3, 26.3, 26.1, 25.2, 24.5.

Dibutyl 3,4,6-Tri-*O*-benzyl-2-*O*-pivaloyl-*β*-D-glucopyranosyl-(1→6)-3,4-*O*-carbonyl-2-*O*-pivaloyl-α-D-galactopyranoside Phosphate 81. General procedure D with donor **1** (76.3 mg, 105 μmol), acceptor **80** (48.3 mg, 0.100 mmol), and TMSOTf (19.4 μL, 105 μmol) afforded 89.0 mg (89%) of **81** as a colorless oil after flash silica column chromatography (40% EtOAc/hexanes). $[\alpha]^{24}_D$ +6.7° (*c* 2.56, CH_2Cl_2); IR (thin film) 2962, 1817, 1740, 1138, 1068 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.37−7.25 (m, 13H), 7.17−7.16 (m, 2H), 5.67−5.64 (m, 1H), 5.12 (d, J = 8.5 Hz, 1H), 5.06−5.02 (m, 2H), 4.77 (d, J = 10.7 Hz, 1H), 4.75 (d, J = 11.0 Hz, 1H), 4.69 (d, J = 11.0 Hz, 1H), 4.58 (d, J = 11.9 Hz, 1H), 4.55−4.46 (m, 3H), 4.44 (d, J = 7.9 Hz, 1H), 4.09−3.99 (m, 5H), 3.86−3.84 (m, 1H), 3.74−3.66 (m, 4H), 3.56−3.52 (m, 1H), 1.67−1.61 (m, 4H), 1.44−1.40 (m, 4H), 1.22 (s, 9H), 1.19 (s, 9H), 0.95−0.91 (m, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 177.1, 176.1, 153.3, 138.1, 138.0, 128.7, 128.6, 128.1, 128.0, 127.9, 127.6, 102.3, 94.1, 83.2, 77.8, 75.3, 75.2, 73.7, 73.2, 71.9, 71.5, 69.4, 68.9, 68.4, 66.9, 39.0, 32.3, 27.3, 27.2, 18.8, 13.8, 13.7; ^{31}P NMR (120 MHz, $CDCl_3$) δ −3.3; ESI MS *m*/*z* $(M + Na)^+$ calcd 1021.4321, obsd 1021.4356.

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Supporting Information Available: Detailed experimental procedures and compound characterization data, including ^{1}H NMR and ^{13}C NMR spectral data for all described compounds and five additional schemes (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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