*Automated Synthesis*

# Automated Solid-Phase Synthesis of Protected Tumor-Associated Antigen and Blood Group Determinant Oligosaccharides**

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The Lewis blood group oligosaccharides are a family of fucosylated, ceramide-containing glycosphingolipids decorating the exterior of healthy and disease-derived cells.[1] These complex carbohydrates consist of a reducing-end lactose β-(1→3) linked to a central *N*-acetyl glucosamine unit. Members of this family of structures differ in the arrangement of galactose and fucose residues around the glucosamine core. Lewis-type penta- and hexasaccharides are part of the inflammatory cascade[2] and have been implicated in bacterial and viral infection,[3] as well as autoimmune diseases.[4] The Lewis Y hexasaccharide and dimeric combinations of Lewis antigens, including the $Le^y$–$Le^x$ nonasaccharide, are tumor markers currently being explored as cancer vaccines.[5]

The biological importance of the Lewis antigens has rendered them targets of intense examination. Since the microheterogeneity of carbohydrates limits the amount of pure oligosaccharides that may be isolated from natural sources, chemical synthesis is essential to procure appreciable quantities of material for biological study. Owing to their significance and complexity, the Lewis blood group determinants have served as synthetic targets for the development of new methods for oligosaccharide synthesis in solution,[6–11] on soluble polymeric supports,[12] and on solid support.[13]

Our strategy for the synthesis of these branched carbohydrates differs markedly from previous routes where maximum convergence was desired. Typically, a series of block couplings specific to each target has been used to combine di- and trisaccharide portions of the molecule. The $Le^y$–$Le^x$ nonasaccharide has been constructed twice in heroic solution-phase total syntheses.[14,15] To simplify the synthesis of such structures, we present here a general method utilizing a small number of common building blocks for a sequential synthesis, by adding one monomer at a time to a growing oligosaccharide chain. With this strategy, access to the entire family of Lewis antigens may be achieved.

Our initial work on automated oligosaccharide assembly demonstrated that relatively simple carbohydrates could be prepared on a machine that executes a coupling cycle, including steps for glycosylation and deprotection.[16] Further fundamental advances are required to expand this methodology to include structures containing a variety of residues and linkages and to simplify the synthetic process.

Initially we identified the monosaccharide building blocks found in the protected Lewis X pentasaccharide **1**, Lewis Y hexasaccharide **2**, and $Le^y$–$Le^x$ nonasaccharide **3** (Scheme 1). Five monomers are sufficient for the construction of the three target structures, which each contain five different glycosidic bonds. Each monosaccharide unit must contain appropriate temporary protecting groups to establish the required connections in the target and be synthetically readily accessible. Additionally, we were interested in monitoring the progress of the solid-phase assembly in real time. The success of individual couplings is routinely monitored during automated oligopeptide and oligonucleotide synthesis.

The differentially protected monosaccharide building blocks were rapidly prepared from glycals by using a one-pot procedure.[17] Glycosyl phosphates **4**, **5**, and **8** were synthesized for the placement of glucose and galactose residues. Glucosamine phosphate **6** was selected based on solution-phase studies. Fucosyl phosphate **7** showed excellent selectivity in creating the difficult α-(1→2-*cis*) and α-(1→3-*cis*) fucose linkages common to the Lewis antigens.[9,17]

For each monosaccharide, a protecting-group pattern that allowed for selective unveiling of a single hydroxy group was selected. We chose the Fmoc group for the temporary protection of the hydroxy groups because it is completely stable under the acidic glycosylation conditions, it is readily cleaved by mildly basic amines,[18] and it facilitates monitoring of protecting-group removal by UV/Vis spectroscopy.[19] Analysis of Fmoc deprotection provided a qualitative assay for the efficiency of each glycosylation and deprotection cycle during automated assembly (Scheme 2). While real-time analysis is common in automated oligopeptide and oligonucleotide synthesis, analogous monitoring of solid-phase glycosylation reactions had not been demonstrated in automated carbohydrate synthesis.[20]

To account for branching connections through both the C3 and C4 positions of the glucosamine units in targets **1**–**3**, an additional temporary protecting group was installed in monomer **6**. The levulinoyl ester was selected, as it is easily removed with hydrazine and is completely orthogonal to the Fmoc group. The use of temporary protecting groups that require only mildly basic conditions for removal allowed the improvement of another feature of previous automated syntheses: the lengthy cleavage of the product from the solid support following assembly. Condensation of the previously employed octenediol linker[21] with carboxy-terminated polystyrene resin resulted in an ester linkage, which was rapidly cleaved with strong base at the end of the synthesis.

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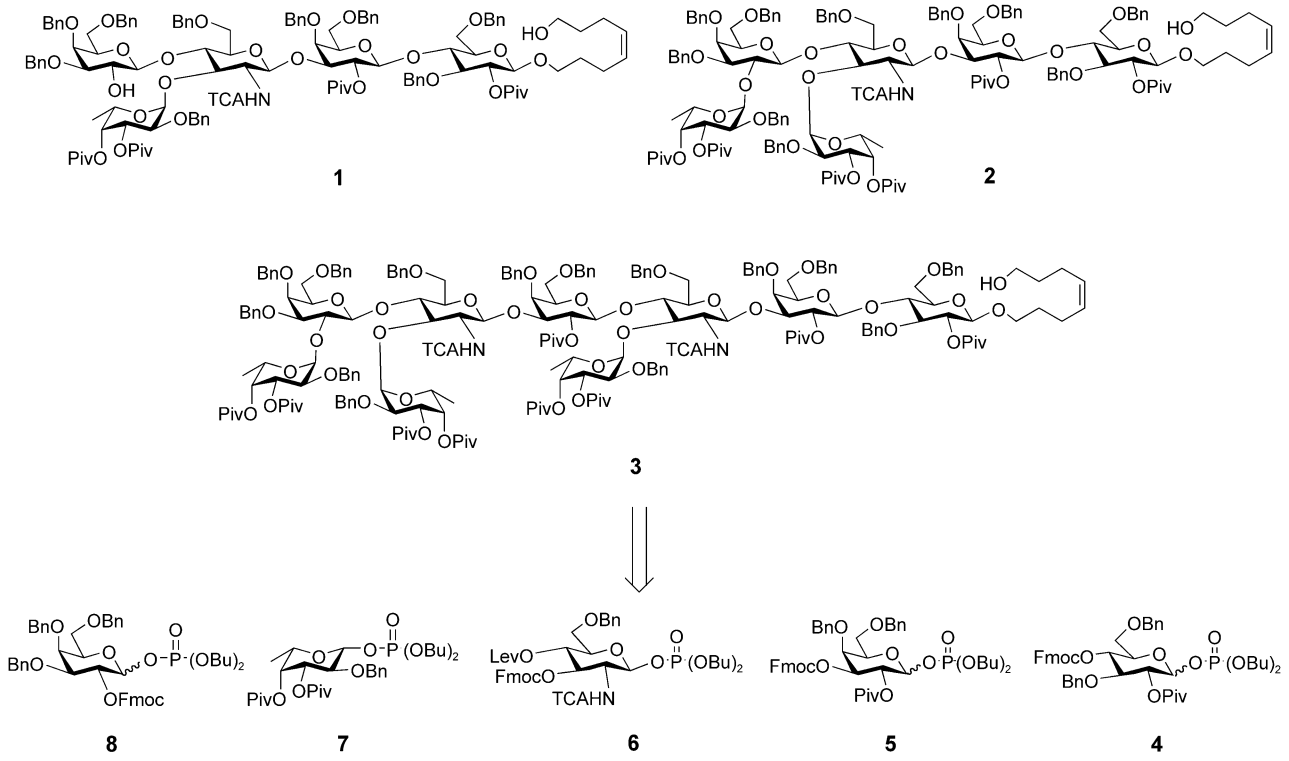
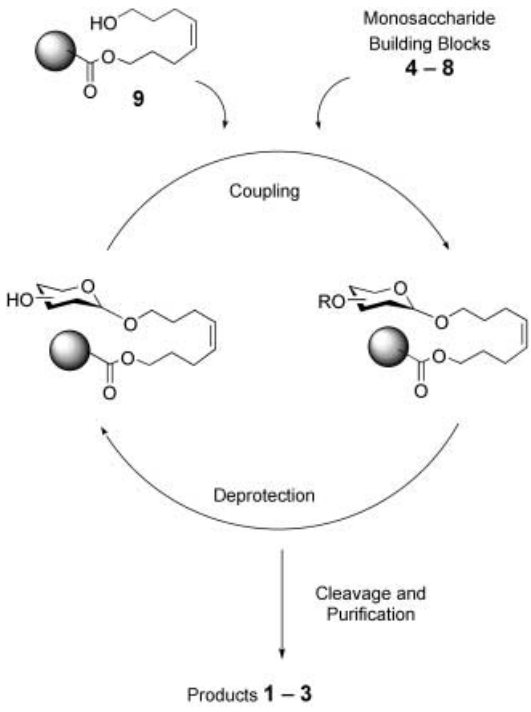
[**] Financial support from the Alfred P. Sloan Foundation (Fellowship to P.H.S.), GlaxoSmithKline (Scholar Award to P.H.S.), Merck, and the National Institutes of Health (NIH; Biotechnology Training Grant Fellowship to K.R.L.) is gratefully acknowledged. Funding for the MIT-DCIF Varian 500 MHz NMR spectrometer was provided by the National Science Foundation (NSF; Award CHE-9808061). Funding for the MIT-DCIF Avance (DPX) 400 MHz NMR spectrometer was provided by the NIH (Award 1S10RR13886-01). Funding for the MIT-DCIF Varian 300 MHz NMR spectrometer was provided by the NSF (Award DBI-9729592). We thank the members of the MIT DCIF for help with the analysis of the reaction products.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

  DOI: 10.1002/ange.200352539 



***Scheme 1.*** Retrosynthesis of the protected Lewis X pentasaccharide **1**, Lewis Y hexasaccharide **2**, and Le$^{y}$–Le$^{x}$ nonasaccharide **3** indicates monosaccharide building blocks **4**–**8**. Bn = benzyl, Bu = butyl, Fmoc = 9-fluorenylmethoxycarbonyl, Lev = levulinoyl, Piv = pivaloyl, TCA = trichloroacetamide.




***Scheme 2.*** Automated oligosaccharide synthesis with glycosyl phosphates. Initial glycosylation of resin-bound acceptor **9** produces a coupling product that may be subsequently deprotected. Iteration of coupling and deprotection cycles with phosphate donors **4**–**8** followed by cleavage of the resin-bound oligosaccharides and purification gives **1**–**3**.

The internal double bond remains a functional handle for further chemistry[21] and can be cross-linked to a variety of olefins by metathesis.

To accommodate real-time monitoring and further increase coupling and deprotection efficiency, new coupling cycles were devised for use on the automated oligosaccharide synthesizer (Table 1). Activation of the phosphate donors was conducted at −15 °C with a solution of TMSOTf. Two sets of deprotection conditions were programmed to effect removal of the Fmoc and levulinoyl ester groups. Fmoc cleavage was achieved by three exposures to piperidine (20 % in DMF). Following each exposure, the solution from the reaction vessel was collected for UV analysis; this enabled determi-

***Table 1:*** General cycle used with glycosyl phosphates for the construction of **1**–**3**.[a]

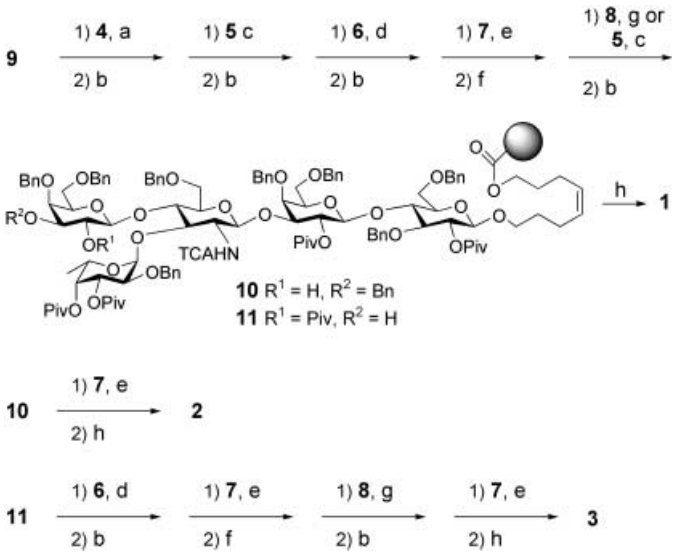
| Step | Function | Reagent | *t* [min] |
|---|---|---|---|
| 1 | coupling | donor (5 equiv), TMSOTf (5 equiv) | 21 |
| 2 | washing | dichloromethane | 9 |
| 3 | coupling | donor (5 equiv), TMSOTf (5 equiv) | 21 |
| 4 | washing | DMF | 9 |
| 5 | deprotection | 3 × piperidine (175 equiv) in DMF or 5 × hydrazine (10 equiv) in DMF | 34 or 80 |
| 6 | washing | DMF | 9 |
| 7 | washing | 0.2 M acetic acid in tetrahydrofuran | 9 |
| 8 | washing | tetrahydrofuran | 9 |
| 9 | washing | dichloromethane | 9 |

[a] TMSOTf = trimethylsilyl trifluoromethanesulfonate, DMF = *N*,*N*-dimethylformamide.

nation of the amount of Fmoc-protected oligomer bound to the solid support at each step. The efficiency of the coupling and Fmoc-removal steps together was resolved qualitatively by using this data, thus allowing termination or correction of syntheses that indicated unproductive coupling steps. Removal of the C4 levulinate group from the glucosamine residue of a resin-bound trisaccharide initially proved to be difficult and required modification of the initial conditions to improve the deprotection efficiencies. The levulinoyl group was removed by five washes with a solution of hydrazine (10% in DMF). A series of washing steps was conducted before and after each deprotection to swell the resin or purge any remaining basic residues in preparation for the next glycosylation cycle.

Protected Lewis X pentasaccharide **1** was constructed in just 12 hours[22] (Scheme 3) by utilizing the reported coupling




***Scheme 3.*** Automated synthesis of pentasaccharide **1**, hexasaccharide **2**, and nonasaccharide **3** on the 25-mmol scale, that is, with 25 mmol of resin (45 mg, 0.56 mmol $g^{-1}$ loading) for each synthesis. a) Donor **4** (5 equiv), TMSOTf (5 equiv), repeated 2 times for 15 min each; b) 20% piperidine in DMF (2.2 mL), repeated 3 times for 10 min each; c) donor **5** (5 equiv), TMSOTf (5 equiv), repeated 2 times for 15 min; d) donor **6** (3.5 equiv), TMSOTf (3.5 equiv), repeated 3 times for 15 min each; e) donor **7** (5 equiv), TMSOTf (5 equiv), repeated 2 times for 15 min each; f) 10% $N_2H_4$ in DMF (2.5 mL), repeated 5 times for 15 min each; g) donor **8** (5 equiv), TMSOTf (5 equiv), repeated 2 times for 15 min each; h) NaOMe (10 equiv) in $MeOH/CH_2Cl_2$ (1:4), repeated 4 times for 90 min each.

cycles (Table 1). Initial glycosylation of linker **9** with glucose monomer **4** was followed by removal of the Fmoc group and iterative addition of monomers **5**–**8** to form the resin-bound pentasaccharide **10**. Cleavage from the support was achieved by repeated treatment with a solution of sodium methoxide over a period of six hours. Pentasaccharide **1** was isolated in 12.6% yield after HPLC purification.

Following our success in synthesizing Lewis X, the reaction sequence was modified to include a coupling cycle for the addition of a fucose residue to the terminal galactose (Scheme 3). In this manner, protected Lewis Y hexasaccharide **2** was synthesized in 14 hours with a yield of 9.9%. The assembly of pentamer **1** and hexamer **2** not only provided access to biologically relevant oligosaccharides but also demonstrated the preparation of important portions of the protected $Le^y$–$Le^x$ nonasaccharide **3**.

The first four monosaccharides of **3** were installed in an identical fashion to the assembly of the Lewis X hexasaccharide **1**. Galactose building block **5** enabled the formation of the additional glucosamine-β(1→3)-galactose linkage. The assembly of **3** (Scheme 3) was completed by addition of glucosamine **6** followed by the final three residues of the Lewis Y hexamer. The solid-phase synthesis of nonasaccharide **3** was finished after 23 hours; cleavage from the solid support and HPLC purification produced **3** as the major product in 6.5% yield.

Two technical advances provided the foundation for rapid access to these complex oligosaccharides of biological importance: monitoring of the monosaccharide installation and a novel linker system. Fmoc analysis proved instrumental in the development of the coupling cycles for all syntheses. Unproductive steps were easily identified and glycosylations or deprotection conditions were modified accordingly to develop successful coupling cycles. Facile removal of the oligosaccharide from the resin by using a base-labile linker enabled swift analysis of the final products. The fully protected Lewis X and Lewis Y oligosaccharides and the $Le^y$–$Le^x$ dimer were all produced in overall yields comparable to or better than previous solution-phase syntheses and in a fraction of the time previously required. Each of these syntheses was completed without initial solution-phase exploration of the synthetic route; monomer reactivity and electronic compatibility with the generated acceptors was tested solely during the course of the automated solid-phase assembly.

Automation of oligosaccharide assembly has the potential to revolutionize the biological understanding of complex oligosaccharides as nonspecialists gain access to a variety of structures. Automated assembly of the $Le^y$–$Le^x$ tumor marker and the Lewis X and Lewis Y blood group antigens by a stepwise monosaccharide approach serves as a first example of our ability to make a host of structures of biological significance. The adaptation of coupling cycles to include Fmoc protection and the introduction of linker strategies to enable base cleavage bring automated methods of oligosaccharide synthesis a step closer to the existing protocols for peptide synthesis. Additional research to expand the diversity of structures accessible through automation, to increase overall yields and to decrease assembly times is on-going.

Received: August 4, 2003
Revised: November 3, 2003 [Z52539]

**Keywords:** automation · carbohydrates · Lewis antigens · oligosaccharides · synthetic methods

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[22] The total time given for the construction of each Lewis oligosaccharide does not include the time required for the synthesis of each monomer building block. While this time cannot be neglected, it should be noted that monomers may be prepared on a large scale and are generally useful in the synthesis of many carbohydrate structures.

  