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HPLC-Assisted Automated Oligosaccharide Synthesis

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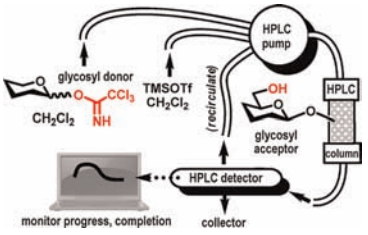
ABSTRACT



A standard HPLC was adapted to polymer supported oligosaccharide synthesis. Solution-based reagents are delivered using a software-controlled solvent delivery system. The reaction progress and completion can be monitored in real time using a standard UV detector. All steps of oligosaccharide assembly including loading, glycosylation, deprotection, and cleavage can be performed using this setup.

Solid-phase synthesis[1,2] has been widely utilized in the routine preparation of oligopeptides[3] and oligonucleotides.[4] The use of polymer supports in oligosaccharide synthesis has also been reported.[5−7] The use of these techniques helps to expedite the synthesis of oligosaccharides and glycoconjugates[8−16] by minimizing the necessity for purifying reaction intermediates and simplifying the removal of excess reagents that is usually achieved by filtration. To expedite solid-phase oligosaccharide synthesis, Seeberger et al. developed an automated approach, which was first accomplished by using a modified peptide synthesizer[17−19] and very recently extended to "the first fully automated solid-phase oligosaccharide synthesizer".[20] Despite being a relatively new technique, it has already been applied to the synthesis of a variety of oligosaccharide sequences.[20−23] Other recent enhancements of the supported synthesis of oligosaccharides include, but are not

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limited to, approaches employing fluorous tag,[24,25] ionic liquid,[26−28] nanoparticle,[29] and nanoporous gold[30] supports.

As a part of an ongoing research effort, presented herein is the development of a new HPLC-based automated synthesis. To obtain clear evidence of the advantages of the new technology in comparison to the state-of-the-art polymer-supported synthesis, we chose the most common approaches for all aspects of our synthesis:[5,7] a solution-based trichloroacetimidate donor, a Tentagel resin-bound glycosyl acceptor attached via the anomeric center, TMSOTf as the activator/promoter, CH_2Cl_2 as the reaction solvent, and Fmoc as the temporary hydroxyl protecting group.[11,16,31]

In accordance with the traditional manual synthesis, a glycosyl acceptor bound to the polymer beads is shaken with an excess of the solution phase glycosyl donor and promoter (Figure 1A). Periodic monitoring of the solution phase by TLC can provide information on whether the glycosyl donor still remains. The completeness of the coupling can be determined experimentally by performing test reactions, such as the Kaiser test,[32] or by cleaving the product off the polymer support followed by characterization. Routinely, the coupling step is repeated two or three times using fresh reagents. Alternatively (or as necessary), the remaining hydroxyls can be capped to ensure that they will not interfere with subsequent steps.

The new setup is based on an unmodified HPLC instrument, which is readily available in practically any synthetic or analytical laboratory. In brief, a chromatography column was packed with the preswelled polymer resin. The column was then connected to the HPLC system containing a pump (a three-head pump was used), a detector (a variable UV range detector), and a computer with standard HPLC-operating software installed (Figure 1B). The column was loaded with the glycosyl acceptor and purged with solvent, and then two separate solutions containing glycosyl donor and promoter were delivered concomitantly. After a relatively short reaction time, typically 30−60 min, the system was purged (washed) with solvent. At this time, the resin is loaded with the disaccharide derivative that can be either cleaved off of the polymer support or the oligosaccharide elongation can be continued via alternating deprotection/glycosylation steps. All steps can be monitored using a standard HPLC detection system set to record changes in the UV absorbance of the solution eluting off the column. A solution of reagents can be recirculated to reduce the amount required for each transformation.

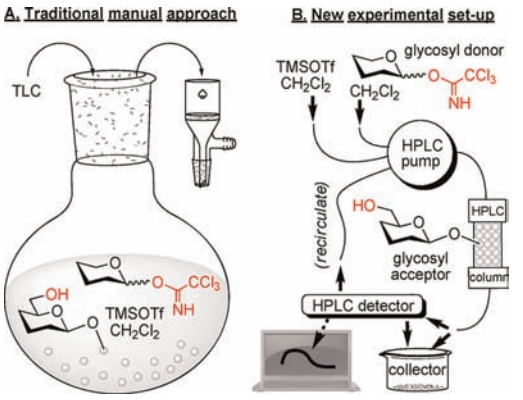

Figure 1. Comparison of the manual polymer-supported synthesis (A) with the new automated setup described here (B).

During our initial experimentation, the attachment of glycosyl acceptor precursor **1a** (3.0 equiv based on the theoretical loading capacity of the resin) to TentaGel MB-NH_2 resin was accomplished using a conventional setup in the presence of EDC (3.0 equiv) and DMAP (1.0 equiv) in CH_2Cl_2. The Kaiser test[32] was conducted on the resin to ensure complete loading (48 h). The resin was then treated with 10% trifluoroacetic acid in wet CH_2Cl_2 to afford polymer bound acceptor **2a** (30 min, loading 0.29 mol/g). Next, resin **2a** (190 mg, 55 μmol of the glycosyl acceptor) was swelled in CH_2Cl_2 for 4−16 h, loosely packed in the Omnifit SolventPlus chromatography column equipped with an adjustable end-piece (http://www.omnifit.com) and the column was integrated into the HPLC.

The relative inefficiency of this protocol motivated us to use the HPLC experimental setup that has allowed us to expedite the initial loading. For instance, loading using a recirculating solution containing **1a** (5 equiv), EDC (5 equiv), and DMAP (1 equiv) in CH_2Cl_2 (Pump B, 1.0 mL/min) was much more effective and the desired loading was achieved in 8 h. After that, the system was purged with CH_2Cl_2 for 10 min (Pump A, 2.0 mL/min flow rate) followed by detritylation that was accomplished using recirculating TFA/CH_2Cl_2/H_2O (10/88/2, v/v/v, Pump C, 1.0 mL/min, 5 min) to afford **2a**. The system was purged with CH_2Cl_2 for 10 min (Pump A, 2.0 mL/min flow rate). Reagent bottles, one containing a 39 mM solution of glycosyl donor **3a**[33] in CH_2Cl_2 and another one containing a 0.28 M solution of TMSOTf in CH_2Cl_2, were connected to inlets for pumps B and C, respectively. Pumps B/C were programmed to deliver the mixed solution of donor/promoter concomitantly in the ratio 4/1 (v/v) at the total flow rate 0.3 mL/min. After 60 min (18 mL total), pumps B and C were stopped and by this time ~10 mol equiv of donor **3a**

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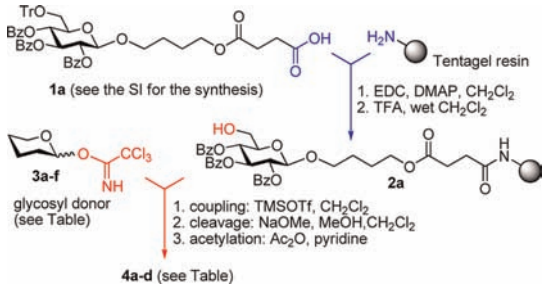
(562 μmol) has passed through the column. It should be noted that the amount of reagents used for this initial study were chosen to mimic that used in the conventional polymer-supported synthesis. Apparently, the flow rate and the donor/promoter ratio can be easily adjusted by simple reprogramming of the pump operation. Since fresh reagents are delivered constantly, this eliminates the need for multiple reiterations of glycosylation, common in manual and other automated techniques. The column was purged with CH_2Cl_2 (pump A, 2.0 mL/min) for 10 min. The formation of disaccharide **4a** was determined by cleaving off the sugar molecule from the resin using a 0.1 M solution of $NaOCH_3$ in CH_3OH/CH_2Cl_2 (5 mL, 1/1, v/v, pump C, 1.0 mL/min, 60 min) carried out by continuous recirculation followed by washing with CH_3OH (2.0 mL/min) for 10 min. Standard (see the Supporting Information (SI)) neutralization and acetylation (Ac_2O/pyridine) afforded product **4a** in 98% yield (Table 1, entry 1).

Encouraged by this result, we explored other donors using essentially the same experimental setup and reaction time. Thus, glycosidation of galactosyl donor **3b**[34] was equally effective and disaccharide **4b** was isolated in 96% yield (Table 1, entry 2). Glycosidations using mannosyl and lactosyl donors **3c**[35] and **3d**[36] were somewhat less efficient, and the resulting oligosaccharides **4c** and **4d** were isolated in 78% and 67% yield, respectively (entries 3 and 4). Finally, glycosidations of glucosyl donors **3e** and **3f** equipped with an easily removable Fmoc protecting group at the C-6 and C-4 positions, afforded **4a** in 95% and 92% yield, respectively (entries 5 and 6). Overall, these results were very indicative of the high efficiency of the new experimental setup based on HPLC, but that generalized reaction conditions (60 min) may not be universally applicable across all sugar series. This result implies that an extended reaction time would be beneficial for driving the glycosidation the less reactive[37–39] of mannosyl donor **3c** to completion, as well as for the reaction with lactose imidate **3d**.

This called for further study, and we noted that the progress of the reaction could be determined from changes in the UV absorbance of the mixture eluting off the column (measured at 254 nm) in comparison to that measured for the initial 0.39 mmol solution of the donor that is entering the column and the expected total absorbance of the donor and TMSOTf (Figure 2A). Once no change is detected (the detector trace reaches a plateau), this can serve as an indication that the reaction had ended. Similar monitoring of the washing stage of the process can provide the desired information about its completion, the trace reaches the baseline corresponding to the standard absorbance of neat CH_2Cl_2.

Real-time monitoring has helped us to optimize the reaction time and reduce the amount of reagents required.

Table 1. Exploratory Comparative Study of the Coupling Efficiency of Glycosyl Donors **3a**–**f** with the Polymer-Bound Acceptor **2a**



entry	glycosyl donor	product	yield
1	**3a**	**4a**	98%
2	**3b**	**4b**	96%
3	**3c**	**4c**	78%
4	**3d**	**4d**	67%
5	**3e**	**4a**	95%
6	**3f**	**4a**	92%

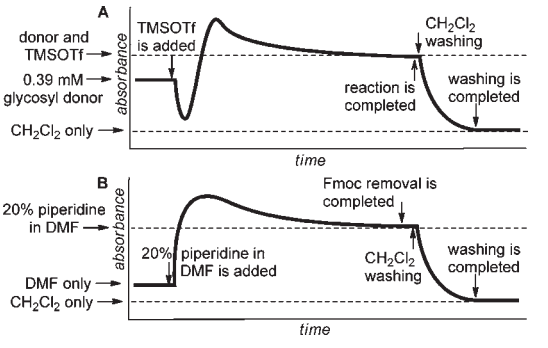


Figure 2. Idealized sketches for the UV detector monitoring: (A) glycosylation–washing steps; (B) Fmoc deprotection–washing.

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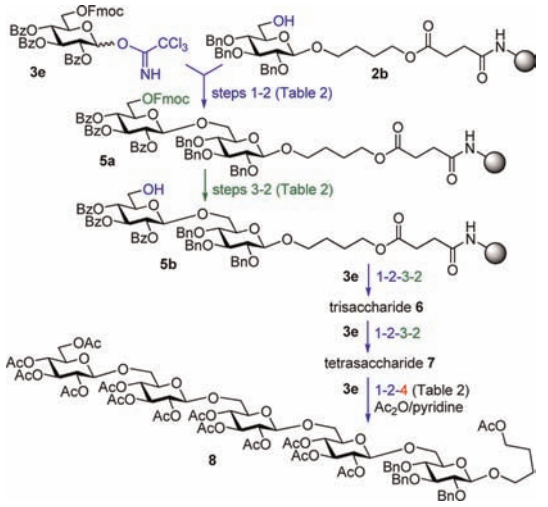
Table 2. Experimental Data for the HPLC-Assisted Synthesis of Pentasaccharide **8**



operation	action	flow rate, mL/min	total volume	time, min
1. Glycosylate (acceptor **2b**)	Pump B: 39 mM **3e** in CH_2Cl_2 Pump C: 0.28 M TMSOTf in CH_2Cl_2	0.3 B/C = 4/1	18 mL	60
2. Wash	Pump A: CH_2Cl_2	2.0	20 mL	10
3. Deprotect **5a**	Pump C: piperidine/ DMF (1/5, v/v)	0.5	2.5 mL	5
2. Wash	Pump A: CH_2Cl_2	2.0	20 mL	10
Repeat 1	As above,	0.3	18 mL	60
2	trisacccharide	2.0	20 mL	10
3	accceptor **6** is	0.5	2.5 mL	5
2	obtained	2.0	20 mL	10
Repeat 1	As above,	0.3	18 mL	60
2	tetrasaccharide	2.0	20 mL	10
3	acceptor **7** is	0.5	2.5 mL	5
2	obtained	2.0	20 mL	10
Repeat 1	Pentasaccharide	0.3	18 mL	60
2	**8a** is obtained	2.0	20 mL	10
4. Cleave off to obtain **8**	Pump C: 0.1 M $NaOCH_3$ in CH_3OH/CH_2Cl_2	1.0	5 mL (recirc.)	60

Thus, the optimized reaction times for glycosylation of acceptor **2a** using a 0.3 mL/min combined flow rate for the donor and promoter solutions (4/1, v/v) are as follows: **3a, e,f** (30 min), **3b** (25 min), **3c** (70 min), and **3d** (85 min). Typical washing times for all reactions at 1.0 mL/min of CH_2Cl_2 are 5−10 min. We also found that slight lowering the concentration of glycosyl donors or performing partial recirculation of the "used" solution by connecting the column outlet with the pump intake (recirculation) is nearly as efficient as using the standard "fresh" 39 mmol solution. However, UV monitoring of experiments wherein reagents are continuously recirculated is cumbersome.

Having optimized model glycosylations we decided to undertake the synthesis of an oligosaccharide chain using the UV detection system. This synthesis began with glycosyl acceptor **2b** and donor **3e** equipped with the temporary Fmoc protecting group. The standard glycosylation protocol (60 min, step 1, Table 2) followed by a 10-min wash (step 2) led to the formation of disaccharide **5a**. Subsequent deprotection of the Fmoc group in **5a** was carried out as follows: purge with DMF for 1 min (2.0 mL/min) and then pass a 20% solution of piperidine in DMF (0.5 mL/min). The release of the dibenzofulvene−piperidine adduct was monitored by the UV detector at 312 nm. Based on the real-time Fmoc deprotection curve (Figure 2B), we were able to reduce the reaction time to 5 min (step 3).

This transition was followed by a 10-min wash with CH_2Cl_2 (step 2) to afford disaccharide acceptor **5b**. The synthesis of trisaccharide **6** was continued using polymer-bound disaccharide acceptor **5b** and donor **3e** via sequential execution of steps 1−2−3−2 (glycosylate−wash−deprotect−wash). The same sequence was repeated to obtain tetrasaccharide **7**. Finally, pentasaccharide was obtained by steps 1 and 2 followed by the cleavage step 4 using a recirculating 0.1 M solution of $NaOCH_3$ in CH_3OH/CH_2Cl_2 followed by acetylation to afford compound **8** in 62% yield. A comparative yield of pentasaccharide **8** was obtained using the manual setup, but the experimental time was significantly longer.

In conclusion, we developed a new technology for automated oligosaccharide synthesis that can now be based on practically any standard HPLC or LC instrumentation. The new technology offers the following advantages in comparison to that of manual oligosaccharide synthesis on polymer supports: faster reaction times, real-time reaction monitoring using an HPLC detection system, and that all steps and sequences can be automated using standard HPLC-managing computer software. This work was greatly inspired by and is complementary to other automated approaches[19,25,40,41] developed for the synthesis of oligosaccharides and related compounds including on-column,[42] flow-through,[24,43] microfluidic,[44] and other related processes.[45,46] Further optimization of the HPLC-based technology and its application to other platforms and targets is currently underway.

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Supporting Information Available. Experimental procedures, extended experimental data, ^1H and ^{13}C NMR spectra for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.