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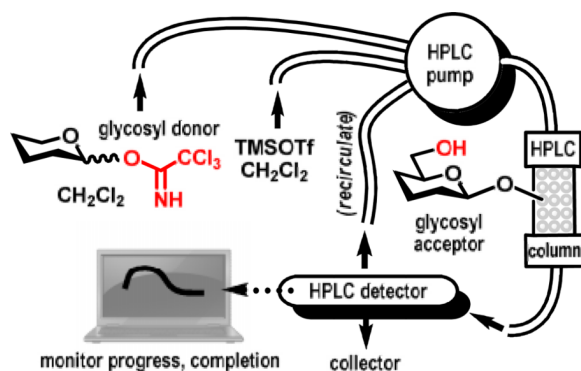
HPLC-Assisted Automated Oligosaccharide Synthesis

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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 (multi-headed pump). The reaction progress and completion can be monitored in real time using a standard variable wavelength UV detector. All steps of oligosaccharide assembly including loading, glycosylation, deprotection, and cleavage can be performed using this experimental set-up.

Solid-phase synthesis^{1,2} has been widely utilized in the routine preparation of oligopeptides³ 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 and co-workers developed an automated approach.^{17–19} The automation was accomplished by using a peptide synthesizer that had been modified in order to be compatible with oligosaccharide assembly using either trichloroacetimidate or phosphate glycosyl donors at low temperature. Although being a relatively new technique, this approach 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 limited to, approaches employing fluororous tag,^{24,25} ionic liquid,^{26–28} nanoparticle,²⁹ and nanoporous gold³⁰ supports.

As a part of the on-going research effort in our laboratories presented herein is the development of a new experimental set-up based on a conventional HPLC for automated

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

oligosaccharide synthesis using insoluble polymer supports. To obtain clear evidence of the advantages of the new technology in comparison to the state-of-the-art polymer-supported synthesis, we deliberately chose the most common approaches for all aspects of our synthesis.^{5,7} Although a vast variety of approaches have been introduced, all of the following are arguably the most commonly seen in the literature: a solution-based trichloroacetimidate donor, a Tentagel resin-bound glycosyl acceptor attached via the anomeric center, TMSOTf as the activator/promoter, CH₂Cl₂ as the reaction solvent, and Fmoc as the temporary hydroxyl protecting group for selective deprotection.^{11,16,31}

In accordance with the traditional manual polymer-bound synthesis, glycosyl acceptor bound to the polymer beads is shaken with an excess of the solution phase glycosyl donor and promoter (Figure 1A). The periodic monitoring of the solution phase by TLC can provide information on whether the glycosyl donor is still remaining in the solution or has been consumed/hydrolyzed. 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 to ensure that all glycosyl acceptor is consumed. Alternatively (or as necessary), the remaining hydroxyls can be capped to ensure that they will not interfere with subsequent steps during the oligosaccharide elongation.

The new experimental set-up is based on an unmodified HPLC, an equipment item that is readily available in practically any synthetic or analytical laboratory. In brief, a chromatography column was packed with the pre-swelled polymer resin. The column was then connected to the HPLC system containing a pump (a three-head pump was used in our experiment), a detector (a variable UV range detector was used herein), and a computer with standard HPLC-operating software installed (Figure 1B). The column was loaded with the glycosyl acceptor, 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.

During our initial experimentation, the attachment of glycosyl acceptor precursor **1a** (3.0 equiv. based on the theoretical loading capacity of the resin) TentaGel[®] MB-NH₂ resin was accomplished using a conventional set-up in the presence EDC (3.0 equiv) and DMAP (1.0 equiv) in CH₂Cl₂. The Kaiser test³² was conducted on the resin to ensure the completion of loading (48 h). The resin was then treated with 10% trifluoroacetic acid in wet CH₂Cl₂ to afford polymer bound acceptor **2a** (30 min). The loading of the resulting resin **2a** was found to be 0.29 mmol/g approximated from all of the following: a) calculation based on the amount of triphenylcarbinol recovered from detritylation; b) recovery of the sugar material from a parallel experiment upon treatment with NaOCH₃/CH₃OH; c) microbalance measurement of the weight difference of resin before and after loading. Next, the preloaded acceptor bound resin **2a** (190 mg, 55 μmol of the glycosyl acceptor) was packed in the Omnifit SolventPlus chromatography column equipped with an adjustable end-piece (<http://www.omnifit.com>). The resin was swelled in CH₂Cl₂ for 4–16 h, loosely packed into the column, and the column was integrated into the HPLC.

The relative inefficiency of this protocol motivated us to use the HPLC experimental set-up 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 CH₂Cl₂ (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 CH₂Cl₂ for 10 min (Pump A, 2.0 mL/min flow rate) followed by detritylation that was accomplished using recirculating TFA/CH₂Cl₂/H₂O (10/88/2, v/v/v, Pump C, 1.0 mL/min, 5 min) to afford the polymer bound acceptor **2a**. The system was purged with CH₂Cl₂ 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 CH₂Cl₂ and another one containing a 0.28 M solution of TMSOTf in CH₂Cl₂ 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 of 4/1 (v/v) at the total flow rate of 0.3 mL/min. After 60 min (18 mL total), pumps B and C were stopped and by this time about 10 mol. equiv. of donor **3a** (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 ratio between the donor and promoter can be easily adjusted by simple reprogramming of the pump operation as needed. The column was purged (washed) with CH₂Cl₂ (pump A, flow rate 2.0 mL/min) for 10 min. The formation of disaccharide **4a** was determined by cleaving off the sugar molecule from the resin using 0.1 M solution of NaOCH₃ in CH₃OH/CH₂Cl₂ (5 mL, 1/1, v/v, pump C, 1 mL/min, 60 min) carried out by continuous recirculation followed by washing with CH₃OH (flow rate 2.0 mL/min) for 10 min. Standard (see the SI) neutralization and acetylation (Ac₂O/pyridine) afforded product **4a** in 98% yield. It should be emphasized that a glycosylation experiment using the conventional experimental set up required significantly longer reaction time (16 h). Additionally, since fresh reagents are delivered constantly, this eliminates the need for multiple reiterations of glycosylation-washing steps as found to be a frequent case for common polymer-bound glycosylations.

Encouraged by this result, we explored other series of glycosyl trichloroacetimidate donors using essentially the same experimental set-up 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, respectively, were practically as efficient as **3a** and disaccharide **4a** was isolated in 95 and 92% yield, respectively (entries 5 and 6). Overall, these results were very indicative of the high efficiency of the new experimental set-up based on HPLC. It came to our attention that generalized reaction conditions (60 min) may not be universally applicable across all sugar series. It has now emerged as common knowledge in carbohydrate chemistry that mannose donors are less reactive than their glucose counterparts.³⁷⁻³⁹ This implies that extended reaction time would be beneficial for driving glycosidation of mannosyl donor **3c** to completion, as well as for the reaction with lactose trichloroacetimidate **3d**.

This called for further study, and it came to our attention that the use of an HPLC detection system would be very informative to indicate when the reaction is nearing completion. For instance, 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. The real-time monitoring would help to optimize the reaction time and, consequently, reduce the amount of glycosyl donor required to drive the reaction to completion. Similar monitoring of the washing stage of the process can provide the desired information about its completion (Figure 2A). In this case, the washing can be considered as

completed once the trace reaches the base-line corresponding to the standard absorbance of neat CH₂Cl₂.

Using this approach, we established that the optimized reaction times for different glycosyl donors for glycosylation with the supported acceptor **2a** using 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 time for all reactions at 1.0 mL/min of CH₂Cl₂ is 5–10 min. We also found that slight lowering of 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 was found to be uninformative.

Having optimized model glycosylations we decided to undertake the synthesis of an oligosaccharide chain using the UV detection system. This synthesis began with glycosyl **2b** and donor **3e** equipped with the temporary Fmoc protecting group. 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 (flow rate 2.0 mL/min) and then pass a 20% solution of piperidine in DMF (flow rate 0.5 mL/min). The release of dibenzofulvene-piperidine adduct was monitored by the UV detector at 312 nm. Based on the real-time Fmoc deprotection curve presented in Figure 2B, we were able to monitor the cleavage process with good accuracy that allowed us to reduce the reaction time to 5 min (step 3).

This transition was also followed by a 10-min wash with CH₂Cl₂ (step 2) to afford disaccharide acceptor **5b**. The synthesis of trisaccharide **6** was continued using polymer-bound disaccharide acceptor **5b** and glycosyl 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 recirculating 0.1 M solution of NaOCH₃ in CH₃OH/CH₂Cl₂ followed by acetylation to afford compound **8** in 62% yield. A comparative yield of pentasaccharide **8** was obtained using conventional experimental set-up, but the experimental time was significantly longer.

In conclusion, we developed a new technology for automated oligosaccharide synthesis. In principle, practically any standard HPLC or LC instrumentation can be used to create a similar set up. The new technology offers the following advantages in comparison to that of conventional 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 the 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 are currently underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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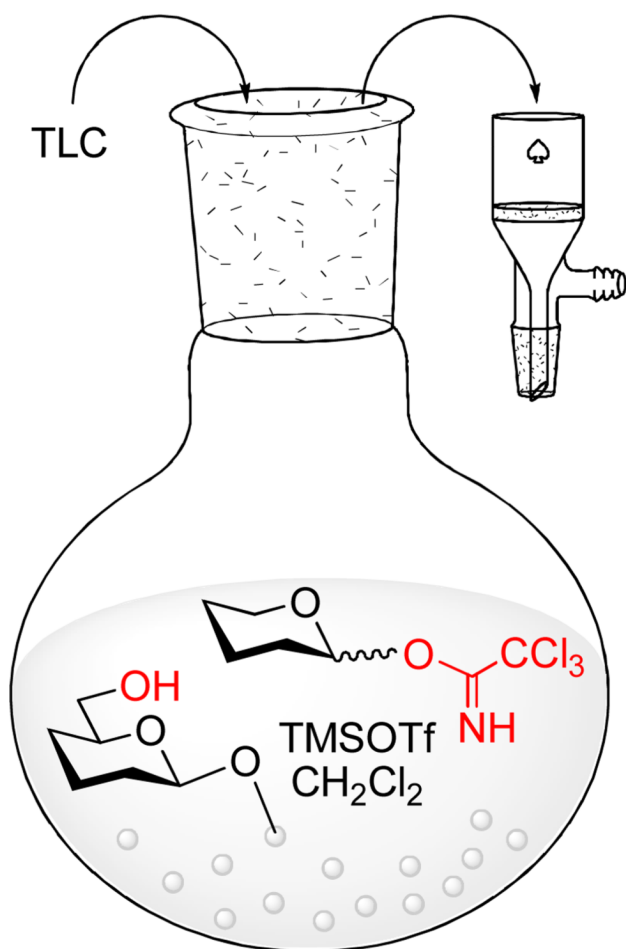
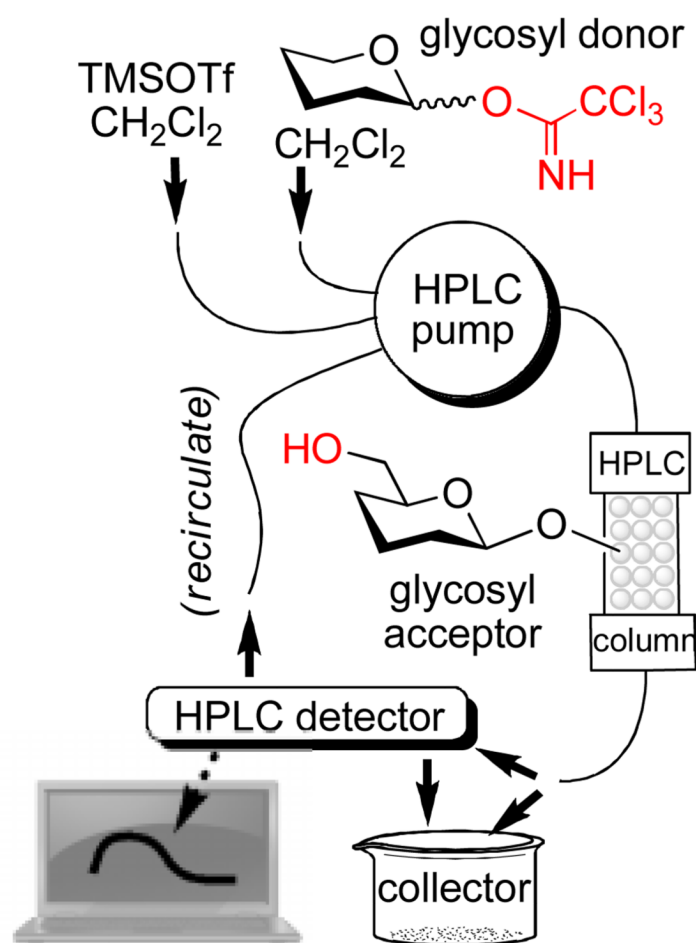
A. Traditional manual approach**B. New experimental set-up**

Figure 1. Comparison of the traditional manual polymer-supported synthesis (A) with the new experimental set-up described here (B).

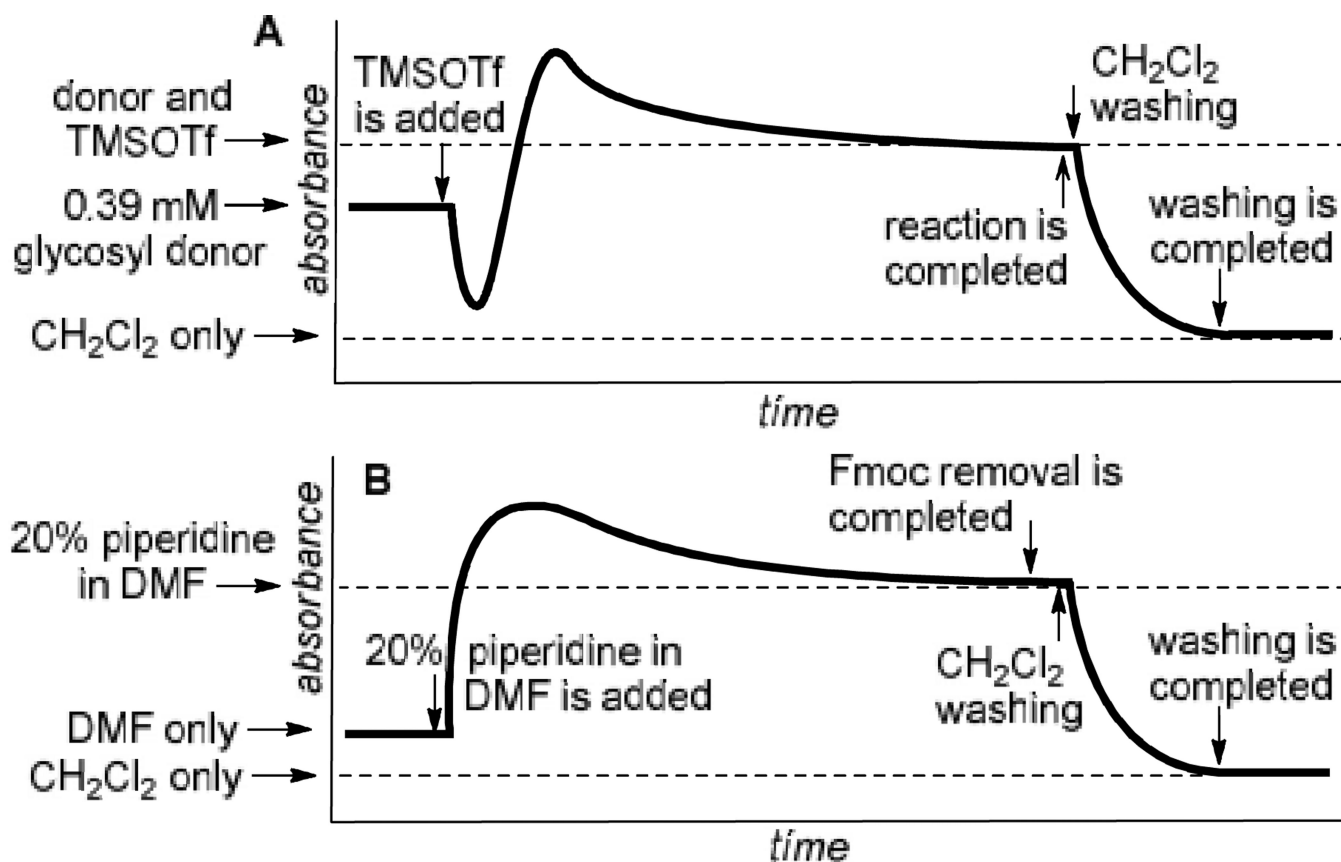
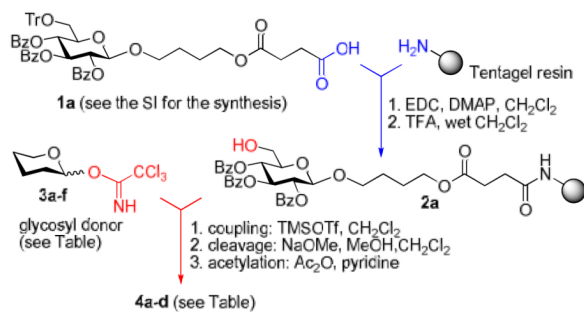


Figure 2. Idealized sketches for the UV-detector monitoring: A. glycosylation-washing steps; B. Fmoc deprotection-washing.

Table 1

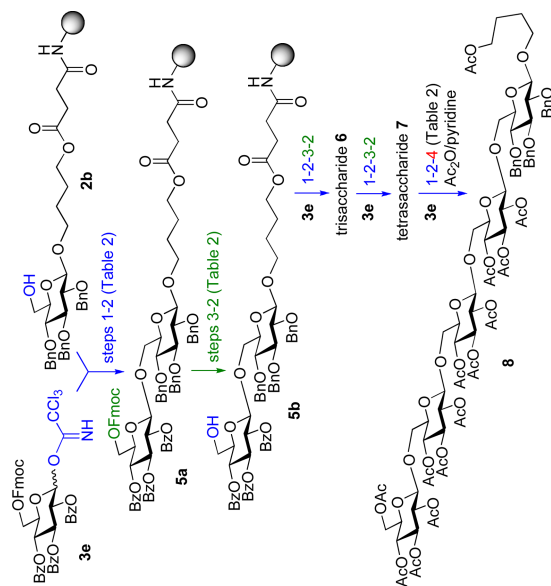
Exploratory comparative study of the coupling efficiency of glycosyl donors **3a-f** with the polymer-bound acceptor **2a**.



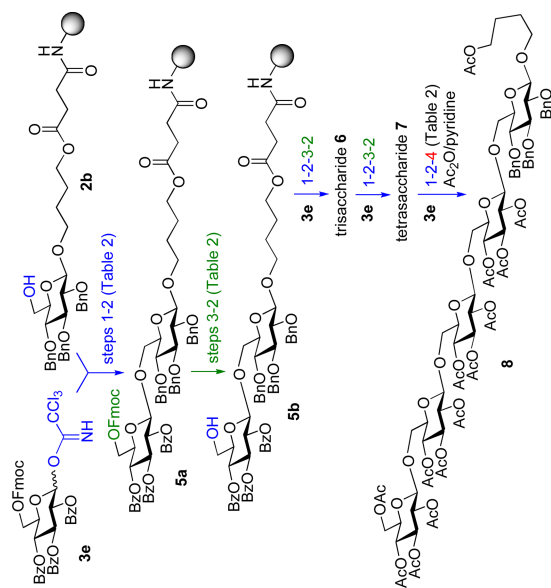
entry	glycosyl donor	product	yield
1	 3a	 4a	98%
2	 3b	 4b	96%
3	 3c	 4c	78%
4	 3d	 4d	67%
5	 3e	 4a	95%
6	 3f	 4a	92%

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 ₂ Cl ₂ Pump C: 0.28 M TMSOTf in CH ₂ Cl ₂	0.3 B/C = 4/1	18 mL	60
2. Wash	Pump A: CH ₂ Cl 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 ₂ Cl ₂	2.0	20 mL	10
Repeat 1	As above, trisaccharide acceptor 6 is obtained	0.3	18 mL	60
2		2.0	20 mL	10
3		0.5	2.5 mL	5
2		2.0	20 mL	10
Repeat 1	As above, tetrasaccharide acceptor 7 is obtained	0.3	18 mL	60
2		2.0	20 mL	10
3		0.5	2.5 mL	5
2		2.0	20 mL	10



operation	action	flow rate, mL/min	total volume	time, min
Repeat 1	Pentasaccharide 8a is obtained	0.3	18 mL	60
2		2.0	20 mL	10
4. Cleave off to obtain 8	Pump C: 0.1 M NaOCH ₃ in CH ₃ OH/CH ₂ Cl ₂	1.0	5 mL (recirc.)	60