VIP Carbohydrates

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Automated Solid-Phase Synthesis of β-Mannuronic Acid Alginates**

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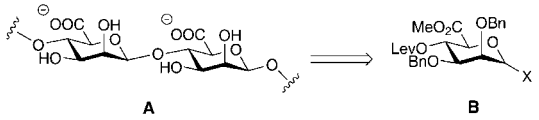
Carbohydrates are the most structurally diverse biopolymers found in nature.[1] This variety is mirrored in the plethora of biological functions that carbohydrates and glycoconjugates perform.[2] Polysaccharides are prime constituents of the cell wall of bacteria and fungi. On the outside of the cell they mediate and modulate the interactions of the cell with its surroundings.[3] As such, many bacterial and fungal polysaccharides are involved in immunological processes. Isolation of these compounds in sufficient amounts and purity to study their biological role in detail is often an impractical task because of the micro-heterogeneity of naturally occurring oligosaccharides. Organic synthesis has the potential to meet the growing demand for well-defined oligosaccharides, including (nonnatural) analogues and conjugates.

Compared to the synthesis of the other biopolymers, the assembly of oligosaccharides is significantly more difficult because the multiple hydroxy groups of the monomeric building blocks have to be discriminated during the synthesis. Perhaps an even bigger obstacle is presented by the union of two saccharides through the formation of a glycosidic linkage, which involves the creation of a new stereocenter.[4] As a direct result, the automated synthesis of oligosaccharide fragments using solid-phase techniques, which has revolutionized nucleotide and peptide chemistry, is still in its infancy.[5] In 2001, Seeberger and co-workers reported on the first automated oligosaccharide synthesizer and showed that oligosaccharide synthesis is amendable to automated synthesis.[6] Key to the success of this methodology is the reliable installation of the desired glycosidic bonds in stereochemically pure form and high chemical yield. The stereoselective construction of 1,2-*trans*-glycosidic bonds nowadays is a routine exercise, both in solution and on the solid support. However, the formation of 1,2-*cis*-glycosidic linkages, especially the β-mannosidic bond, still presents a significant synthetic challenge.[4] The most powerful solution for the β-mannoside problem reported to date was developed by Crich and co-workers,[7] who showed that benzylidene-acetal-protected mannosides could be used for the formation of the 1,2-*cis*-mannosyl linkage. However, transposing the benzylidene mannosylation chemistry to the solid support was met with varying success.[8,9]

Herein we report on the automated solid-phase assembly of mannuronic acid alginate oligomers, featuring up to twelve 1,2-*cis*-mannosidic linkages. The structures were constructed using a second-generation automated oligosaccharide synthesizer[10] and the stereoselective formation of the β-mannosidic linkages was secured through the use of mannuronic acid donors. The use of the synthesizer allowed us to rapidly access target structures, without intermediate purification and in quantities that are not only sufficient to cater for biological experiments but also to facilitate verification of the structural integrity of the compounds using standard ^{1}H and ^{13}C NMR techniques.

Poly-β-(1,4)-mannuronic acid alginate (**A**, Scheme 1) is a major component of the cell wall of various algae.[11] It also represents the exo-polysaccharide of *Pseudomonas aeruginosa*,[12,13] an opportunistic, nosocomial gram-negative bacterium, which poses a serious health threat to immunocompromised patients. Small mannuronic acid oligomers have been shown to have Toll-like receptor 2 and 4 mediated immunomodulatory activity.[14] To enable the study of the antigenicity and immunomodulatory effects of mannuronic acid alginates we recently started a synthetic campaign aimed at their construction.[15] During these studies we discovered that mannuronic acid donors are highly β-selective glycosylating agents. Using solution-phase chemistry we were able to assemble a mannuronic acid pentamer using a convergent synthetic strategy, in which a monomeric acceptor was elongated with two successive disaccharide donors in 51% and 69%, respectively.[15a] We reasoned that an automated solid-phase synthesis approach could be more efficient for the assembly of a library of larger mannuronic acid alginate



A **B**

Scheme 1. Target structure β-(1,4)-mannuronic acid alginate (**A**), which is synthesized using mannuronic acid building blocks **B** (X = SPh, OC(=NPh)CF_3). Bn = benzyl, Lev = levulinoyl.

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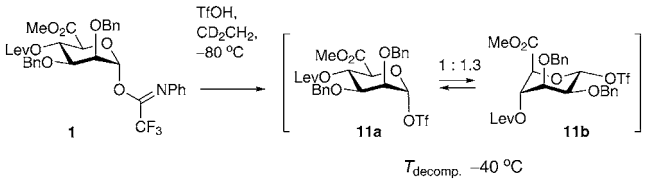
fragments. The success of this approach clearly hinges on the efficient construction of the β-mannuronic acid bonds, which have to be introduced in high yield and in a stereoselective manner to prevent the formation of inseparable (anomeric) mixtures. In our solution-phase experiments we generally use thioglycosyl donors in a preactivation glycosylation protocol. Since preactivation of the donors is not (yet) possible on the synthesizer and the nature of the linker prohibits the use of soft electrophiles, required for the activation of thioglycosides, we chose to use *N*-phenyltrifluoroacetimidate[16] donor **1** as the key building block (Scheme 2). This donor can be

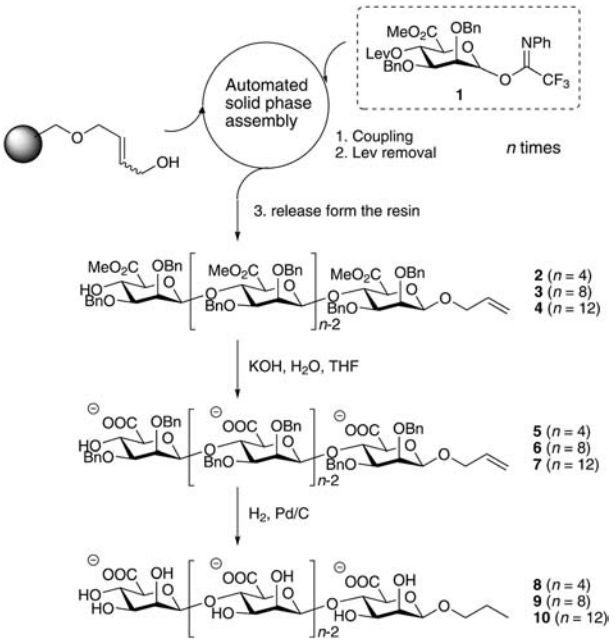


Scheme 2. Investigation into the activation of glycosylating agent **1** using low-temperature NMR spectroscopy. Tf = trifluoromethanesulfonyl.

prepared from known thioglycoside intermediates on a multigram scale,[17] and is activated by a catalytic amount of Lewis or Brønsted acid. The levulinoyl ester was installed as a temporary protecting group at C4 because this can be selectively cleaved under near-neutral conditions without touching the methyl esters or causing epimerization or β elimination.

The activation of **1** was first examined in a low-temperature NMR experiment (Scheme 2). To this end **1** was dissolved in [D_2]dichloromethane and treated with a slight excess of TfOH at −80 °C. The donor was rapidly consumed to provide a conformational mixture of the two anomeric α-triflates **11a** and **11b**, as we previously established for the analogous thiophenyl donor (**B**-SPh; Scheme 1).[18] The excess of TfOH in this experiment proved to be too acidic for the intermediate triflate **11**, thus resulting in degradation of the mixture. As a comparison, when the corresponding thiophenyl donor was activated using 'neutral' conditions (Ph_2SO/Tf_2O), the same anomeric triflate was produced. Under these reaction conditions the temperature of decomposition of triflate **11** was determined to be −40 °C. This relatively low decomposition temperature provides an indication of the reactivity of the donors at hand, which is of importance in establishing optimal glycosylation conditions (see below).

We then moved to the automated solid-phase synthesizer to investigate the chemistry on solid support. Merrifield resin[19] was functionalized with a butenediol linker (loading 0.34 mmol g^{-1}), which allows cleavage of the products from the solid support through cross-metathesis with ethylene.[20] Before the assembly of the larger oligomers, we started off with optimizing the coupling and deprotection steps. In a first attempt, **1** (2 × 5 equivalents)[21] was coupled to the resin under the agency of a catalytic amount of TMSOTf (0.2 equiv with respect to **1**) at 0 °C. Cleavage of a sample from the resin by cross-metathesis with ethylene using Grubbs' first-generation precatalyst gave a mixture of anomeric diastereomers (α/β = 1:3). Although the β product was predominantly formed, the stereoselectivity was clearly insufficient to be used in the assembly of larger oligomers. We therefore lowered the reaction temperature of the glycosylation reaction close to the decomposition temperature of the intermediate triflate (−40 °C) to assure its intermediacy in the reaction. This step resulted in the exclusive formation of the β-linked product, as judged by ^{1}H NMR spectroscopy of the sample mixture that was cleaved from the resin. For the removal of the C4-O-levulinoyl ester optimal reaction conditions were found in the use of H_2NNH_2·HOAc (10 equiv) in a mixture of pyridine/AcOH (4:1 *v*/*v*) at slightly elevated temperature (40 °C). Finally the coupling efficiency in terms of monomer to dimer conversion was optimized. It was found that glycosylating with two coupling cycles of **1** (5 equiv) and TMSOTf as a promotor led to a conversion of approximately 80 %. Changing to a protocol in which the TfOH was used as an activator and the coupling cycle was repeated three times with 3 equivalents of **1**, led to a significantly better conversion (> 95 %). Also, the reaction mixtures were collected after each coupling, which allowed recovery of the unreacted **1** in approximately 20 %. Separate automated synthesis runs were conducted to generate tetrasaccharide **2**, octasaccharide **3**, and dodecasaccharide **4** (Scheme 3, and see the Supporting Information).



Scheme 3. Automated solid-phase assembly of mannuronic acid alginates.

In a generalized procedure, the reaction vessel of the synthesizer was charged with resin (100 mg, 34 μmol) and this was subjected to the number of coupling/deprotection cycles as programmed. After the final deprotection step, the resin was collected, the products were released from the resin by

metathesis, and the crude reaction mixture was analyzed by LC/MS and NMR spectroscopy. As can be estimated from the ELSD trace of the LC chromatogram,[17] the crude reaction mixture of the tetramer synthesis contained about 90% of the desired product **2**, next to a minor amount of the deletion sequence trisaccharide, thus indicating a high coupling efficiency. Importantly, the NMR spectra of the crude cleavage mixture showed that the coupling reactions had proceeded with excellent stereoselectivity. The relatively high chemical shifts of the anomeric signals in the ^{13}C APT spectrum are indicative of β-mannosidic linkages. Furthermore, the heteronuclear one-bond C_1-H_1 coupling constants ($J_{C1\text{-}H1} \approx 156$–$158$ Hz) unambiguously ascertain the installation of the 1,2-*cis*-linkages. The construction of longer fragments proceeded equally well. The automated solid-phase synthesis of octamer **3** led to a crude reaction mixture containing approximately 55% of the desired product, which equals about 90% efficiency per coupling cycle. Dodecamer **4** made up approximately 40% of the crude reaction mixture obtained after 12 repetitive coupling/deprotection cycles as indicated by LC/MS (Figure 1A), thus representing about 90% coupling efficiency. Interestingly, the ^1H and ^{13}C APT spectra of the crude reaction mixtures obtained from compound **3** and **4** are remarkably similar to the spectrum obtained for the reaction mixture from **2**, and only differ in the intensity of the signals belonging to the internal mannuronic acid residue.[17] This data indicates that the structures of the oligomers are very regular, and that the glycosidic bonds have been introduced with excellent β stereoselectivity.

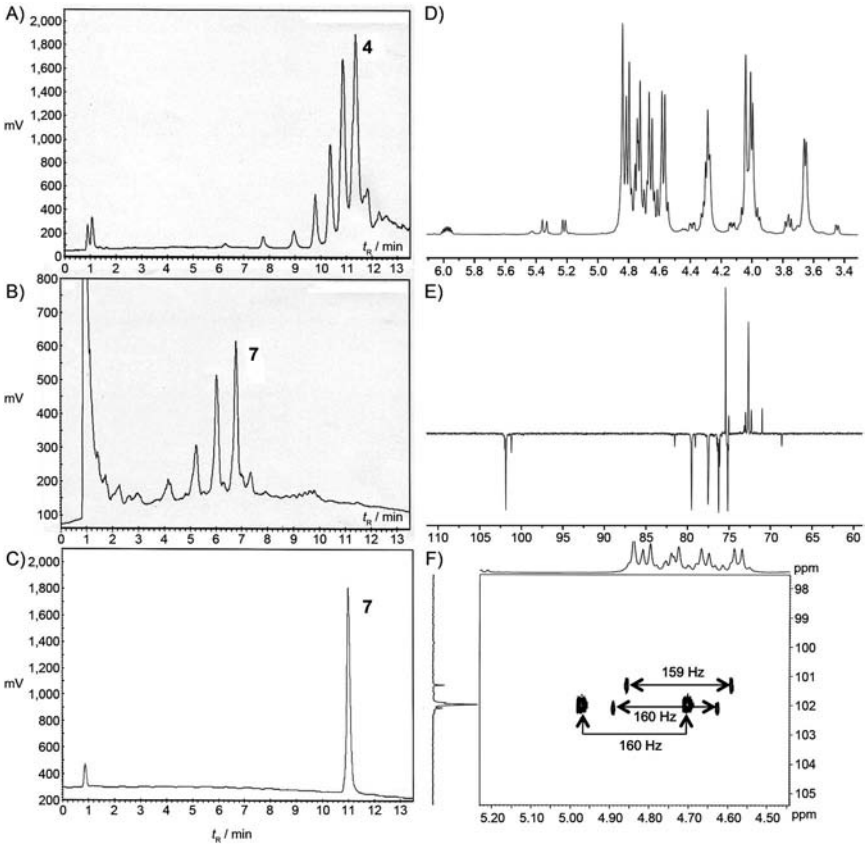


Figure 1. A) LC/MS chromatogram of the crude reaction mixture containing dodecasaccharide **4** (gradient 70→95%); B) LC/MS chromatogram of the saponified mixture containing dodecasaccharide **7** (gradient 70→95%); C) LC/MS chromatogram of purified product **7** (gradient 50→90%); D) ^1H NMR spectrum of saponified product **7** after HPLC purification; E) ^{13}C APT NMR spectrum of pure product **7**; F) HMBC-GATED NMR spectrum of the pure product **7**.

Preparative HPLC purification of the oligomers was accomplished after saponification of the methyl esters (KOH, THF/H_2O, 1:1, v/v), because at this stage an improved baseline separation of the peaks corresponding to the target products in the HPLC trace was observed (Figure 1B). In this way tetramer **5** was obtained in 24 mg, octamer **6** in 20 mg, and dodecamer **7** in 17 mg (NMR analysis of product **7** is shown in Figures 1D–F). The amounts of mannuronates isolated correspond to overall yields of 47% for tetramannuronate **5** (8 on-resin steps), 16% for octamannuronate **6** (16 on-resin steps), and 11% for dodecamannuronate **7** (24 on-resin steps).[22] These numbers approach a yield of 90% per chemical step. Final deprotection of the partially protected oligomers was accomplished by hydrogenolysis over Pd/C in THF/H_2O/*t*BuOH (1:1:1, v/v/v) to provide the target tetramer **8**, octamer **9**, and dodecamer **10** in excellent yields and multimilligram quantities.

In conclusion, we have described the automated synthesis of mannuronic acid alginates featuring up to twelve 1,2-*cis*-mannosidic bonds, using a second-generation oligosaccharide synthesizer. It has been shown that the synthesizer is capable of rapidly delivering oligosaccharides of a length difficult to obtain by solution-phase techniques. Importantly, the multimilligram quantities of the compounds are not only sufficient for biological experiments but also enable the full structural characterization of the compounds. Together with the recent advances in the stereoselective construction of 1,2-*cis*-glucosidic and 1,2-*cis*-galactosidic linkages,[23] this represents an important step forwards towards routine automated solid-phase oligosaccharides assembly. Our growing insight into the mechanisms of glycosylation reactions in combination with the future availability of commercial synthesizers and ever more powerful purification techniques will open up new avenues in glycochemistry and glycobiology.

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[21] Because of the high overall yield of an automated solid-phase synthesis campaign, the synthesis can be executed at a significantly smaller scale than the analogous solution-phase synthesis. As a result the amount of building block required is not as big as often assumed. Depending on the efficiency of both the solution-phase and solid-phase processes, the latter can in fact be more "building block economical" than the former.
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