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Automated Solid-Phase Synthesis of Hyaluronan Oligosaccharides

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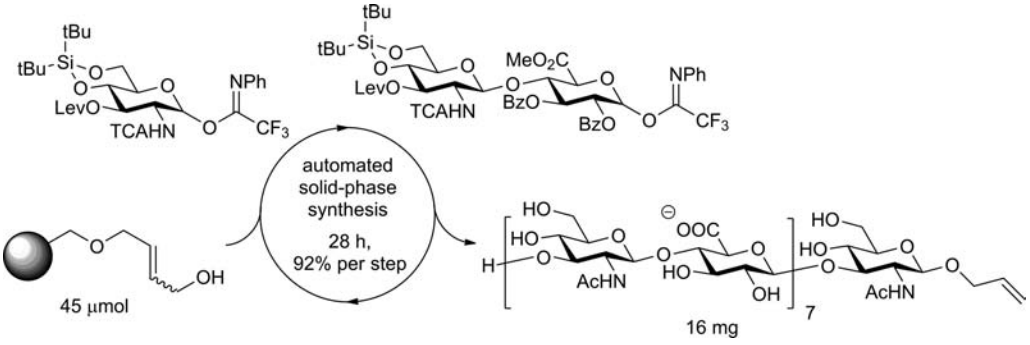
ABSTRACT



Well-defined fragments of hyaluronic acid (HA) have been obtained through a fully automated solid-phase oligosaccharide synthesis. Disaccharide building blocks, featuring a disarmed glucuronic acid donor moiety and a di-*tert*-butylsilylidene-protected glucosamine part, were used in the rapid and efficient assembly of HA fragments up to the pentadecamer level, equipped with a conjugation-ready anomeric allyl function.

Hyaluronan, or hyaluronic acid (HA), a member of the glycosaminoglycan (GAG) family, is composed of [→4)-β-D-Glc*p*A-(1→3)-β-D-Glc*p*NAc-(1→] tandem repeats reaching up to 10^4 disaccharides (~3.7 × 10^6 Da) in length.[1] HA is a major component of the extracellular matrix, connective tissue, and synovial fluid in mammals and also occurs in capsules of certain bacteria. Besides functioning as a molecular lubricant, HA plays an important role in many biological processes, including inflammatory response, cellular proliferation, cell−cell recognition, cell migration, and cell adhesion.[1−3] These processes depend on interactions with a variety of HA binding proteins on the cell surface or in the extracellular fluid. One of the most prominent examples of HA-binding proteins is CD44, a transmembrane receptor present on leukocytes, fibroblasts, endothelial cells, and epithelial cells, which is involved in many processes including lymphocyte recruitment, T-cell signaling, apoptosis, and tumor metastasis.[4] The mode of action of HA fragments has been shown to depend on its length. For example, whereas long HA chains are immunosuppressive, small HA fragments are immunostimulatory and function as an endogenous danger signal.[1,5,6] In the same vein, long HA stretches are required for the formation of CD44 signaling

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complexes that are thought to govern cancer cell proliferation, where small HA fragments appear to act as CD44 antagonists.[1,2,7] HA fragments with a minimum size of six monosaccharides can bind to CD44, and a HA 10-mer effectively competes for binding with full length HA.[8]

To study HA−protein interaction, the availability of well-defined HA fragments is a prerequisite, and therefore, the synthesis of HA has been actively pursued. Various strategies have been developed toward its assembly,[9] including enzymatic[10] and chemical methods involving both postglycosylation[11] and preglycosylation oxidation approaches,[12] and one-pot procedures.[13] Recently, the first studies toward soluble polymer-supported syntheses have been described.[14] Chemical solution-phase synthesis has provided access to well-defined HA fragments composed of two to ten monosaccharide residues.[12e] Application of a soluble polymer support has delivered an HA-dimer.[14]

The repetitive nature of the HA polymer invites the assembly of well-defined oligomers by means of an automated solid-phase approach.[15] However, and as opposed to the automated synthesis of oligopeptides and oligonucleotides, the automated solid-phase synthesis of carbohydrates is not yet a routine operation and is hampered by the lack of a standard set of carbohydrate building blocks and coupling chemistry. A major challenge in the assembly of HA, and GAGs in general, is the low reactivity of the building blocks required.[16] Previous work on the soluble polymer-supported assembly of HA and heparin fragments has made it clear that the translation of a solution-phase synthesis to the (solid) support is not a trivial operation.[17] Solid-phase synthesis approaches (including automated protocols), however, have the intrinsic advantage that coupling reactions can be forced to completion by the use of excess reagents and repetitive coupling cycles. Although repetitive cycles and excess reagent indicate the need for significant amounts of building blocks, the fact that the overall assembly can be high-yielding allows one to start an assembly sequence on a relatively small scale, which would make the process in fact quite building block efficient.[15c] This holds true especially for higher oligomers composed of repeating (mono)saccharides, such as present in HA. We now describe the first automated synthesis of a set of HA oligomers up to the pentadeca level. Our work entails the first example of the construction of well-defined short- and medium-sized GAG oligomers using an automated solid-phase carbohydrate synthesis protocol.

Our approach is based on the use of Merrifield resin, functionalized with a butenediol linker system[15a] in combination with a monomeric glucosamine synthon (**1**),[12d] and the repetitive use of an orthogonally protected GlcNHAc-GlcA building block (**5**, Scheme 1). The butenediol linker is inert to all reaction conditions used during the assembly of the oligomers and can be cleaved through a cross-metathesis reaction to deliver an anomeric *O*-allyl functionality. Because of the protecting group scheme devised, the anomeric allyl group can be retained until the end of the synthesis and immediately serve as a ligation handle.[18] The glucosamine and dimer building blocks **1** and **5** feature a di-*tert*-butylsilylidene ketal to mask the C4- and C6-hydroxyl functions. This protecting group has been selected because of its excellent acid stability, which is of prime importance given the fact that repetitive glycosylations are performed using relatively large amount of Lewis acid (with respect to conventional solution-phase conditions). The influence of the amine-protecting group in building block **1** was assessed in a series of model glycosylation reactions (Supporting Information). From the protecting groups scrutizined (trichloroacetyl, trifluoroacetyl, trichloroethoxycarbonyl, and benzyloxycarbonyl), the trichloroacetyl-protected glucosamine **1** emerged as the most productive donor of the series. With this donor, the key disaccharide building block **5** was assembled as depicted in Scheme 1. In a chemoselective glycosylation reaction, *N*-phenyl trifluoroacetimidate donor **1** was condensed with *S*-phenylglucuronic acid **2** (obtained from D-glucose in seven steps through solely crystalline intermediates, see the Supporting Information) to furnish dimer **3**,[12d] which was transformed into imidate donor **5** through hydrolysis of the thioacetal and installation of the *N*-phenyltrifluoroacetimidate function.[19,20] Following this approach, building block **5** was readily assembled on a multigram scale.

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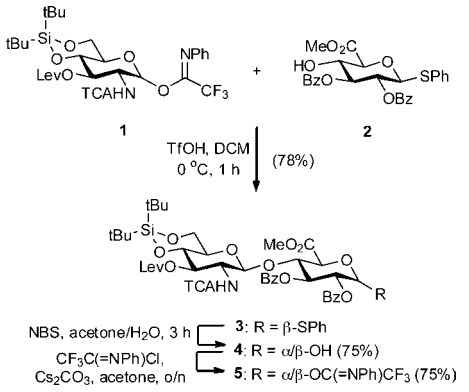
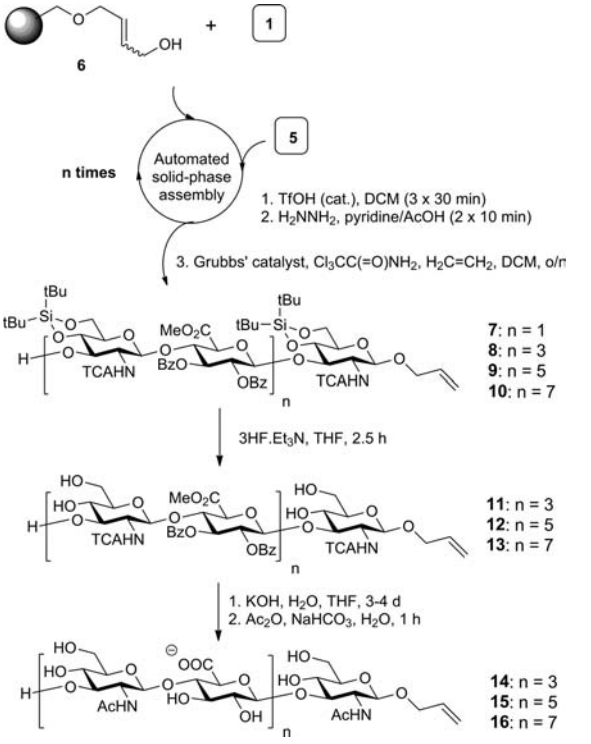
Scheme 1. Synthesis of Disaccharide Building Block **5**



At the onset of the solid-phase assembly of the HA oligomers, it became clear that the disaccharide synthon **5** could not be used as the first building block,[21] and we therefore started our automated syntheses with the coupling of glucosamine **1** on the solid support (**6**). To probe the two building blocks, the on-resin deprotection steps, and the efficacy of the cross-metathesis cleavage reaction we initially assembled trisaccharide **7** (Scheme 2). Treatment of resin **6** with building block **1** (2.7 equiv donor, 0.33 equiv TfOH, DCM, 0 °C, repeated three times) was followed by levulinoyl removal (H_2NNH_2.HOAc, 7.8 equiv, pyridine/AcOH, 40 °C, repeated twice) (see the Supporting Information for detailed reaction conditions). Next, a similar reaction sequence using building block **5** led to the resin-bound trisaccharide. Upon cleavage from the resin the crude trisaccharide **7** was obtained in 90% yield. LC–MS and NMR analysis of this trimer revealed that both glycosylation reactions had proceeded with excellent stereoselectivity and that no deletion sequences or orthoester side products were formed. The only observable side product produced in this sequence of reactions proved to be a trisaccharide of which one of the TCA groups was transformed into a dichloroacetyl group (~5%). In the projected automated synthesis of higher HA oligomers, partial conversion of some of the TCA protecting groups to a DCA would significantly hinder characterization and purification of the products, and in addition, harsher conditions would be required for the removal of the DCA groups at the end of the synthesis. Importantly, these problems would increase drastically with the growing length of the desired oligosaccharides. Dehalogenation of the TCA functionalities could be the result of the nucleophilic attack of a tricyclohexylphosphine ligand of the Grubbs catalyst on one of the chlorine atoms, and we explored several possibilities to circumvent this side reaction.[22] The use of different metathesis catalysts was to no avail, and we therefore switched to the use of a "decoy" substrate in the cleavage reaction. The addition of an excess trichloroacetamide to the metathesis reaction proved effective and completely suppressed the side reaction. With these optimized reaction conditions in hand we set out to assemble hepta-, undeca-, and pentadecasaccharidic fragments of hyaluronic acid, as depicted in Scheme 2. The three syntheses all commenced with glycosylation of resin **6** with glucosamine donor **1** and subsequent Lev-deprotection. Three coupling/deprotection cycles with disaccharide donor **5** gave heptasaccharide **8**, five coupling/deprotection cycles with **5** gave undecasaccharide **9**, and seven coupling/deprotection cycles with **5** provided pentadecasaccharide **10**.[23]

Scheme 2. Automated Solid-Phase HA-Assembly



LC–MS analysis of the crude heptamer **8** (Supporting Information) indicated that the synthesis had proceeded very efficiently and only a minor deletion sequence was detected (ratio pentamer/heptamer ~1:80). The undeca- and pentadecamers **9** and **10** proved to be too lipophilic for HPLC analysis, but MALDI mass spectroscopy confirmed the presence of the target compounds in the crude reaction mixtures. Partial deprotection of the oligomers by cleavage of the silylidene ketals led to more hydrophilic compounds, which could be analyzed and readily purified by HPLC as depicted in Figure 1. Although there is relatively little experience in the HPLC purification of protected oligosaccharides, these results indicate that a strategy that allows for the partial deprotection of the material

(21) The combination of a reactive primary alcohol acceptor and a glucuronic acid donor bearing a C-2 acyl protecting group can give rise to acyl migration in the course of the glycosylation reaction. For this type of migration, see: Bérces, A.; Whitfield, D. M.; Nukada, T.; do Santos Z., I.; Obuchowska, A.; Krepinsky, J. J. *Can. J. Chem.* **2004**, *82*, 1157–1171. Also see ref 12a.

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(23) During the pentadacamer assembly, the reaction mixtures and subsequent DCM washes were collected, from which 37% of unreacted donor **5** was recovered.

that is released from the resin, such as described here, delivers intermediates of such a polarity that they are readily amendable to routine HPLC purification. In this way, heptasaccharide **11** was isolated in 26% over 10 steps (~87% per step), undecasaccharide **12** in 32% over 14 steps (~92% per step), and pentadecasaccharide **13** in 18% over 18 steps (~91% per step), starting from 45 μmol of functionalized resin **6**.

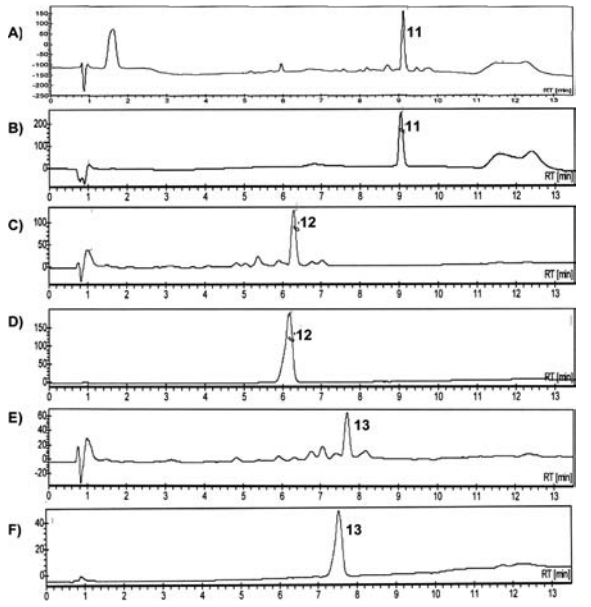

Figure 1. LC-traces of heptamer **11**, undecamer **12**, and pentadecamer **13** prior to (A, C, D) and after (B, D, F) HPLC purification.

Global deprotection of the compounds was accomplished by saponification of the benzoate esters, methyl esters, and trichloroacetamides by treatment with 0.5 M KOH in a mixture of THF/H_2O to provide the "zwitterionic" hepta-, undeca-, and pentadecamers, which were purified by gel permeation chromatography. The pentadecamer proved to be relatively poorly soluble in water, and the addition of a few drops of aqueous ammonia was required to fully solubilize the compound. Finally, the amine groups in the target compounds were acetylated to provide the hyaluronic acid fragments **14**, **15**, and **16**. As an indication of the overall efficiency, the single solid-phase run of the pentadecamer required 28 h for the assembly of the fully protected intermediate and delivered 16 mg of the pure, allyl-functionalized final compound, the integrity of which was fully ascertained by ^{1}H and ^{13}C spectroscopy as depicted in Figure 2. The relatively simple NMR spectra indicate that the HA-pentadecamer takes up a very regular structure.

In summary, we have described the first automated solid-phase synthesis of a set of hyaluronic acid oligomers, representing the first GAG oligomers to be synthesized in a fully automated fashion. The synthesis strategy is based on the combined use of mono- and dimeric building blocks and proceeded both rapidly and efficiently. The disaccharide building block, which was used for the repetitive elongation cycles, represents a disarmed donor glycoside, indicating that low reactivity of glycosyl donors presents no obstacle for the automated solid-phase assembly platform. The protecting group scheme devised for the synthesis not only led to an effective assembly process but also allowed for the incorporation of a conjugation-ready allyl functionality on the anomeric center through the use of a butenediol linker system in combination with an optimized cross-metathesis cleavage reaction. The reported solid-phase assembly indicates that the automated solid-phase synthesis of other members of the glycosaminoglycan family is within reach. Because of the modular nature of the GAG structures, automated synthesis is a very attractive technique to assemble (libraries of) well-defined GAG fragments, making these important saccharides available for biological studies.

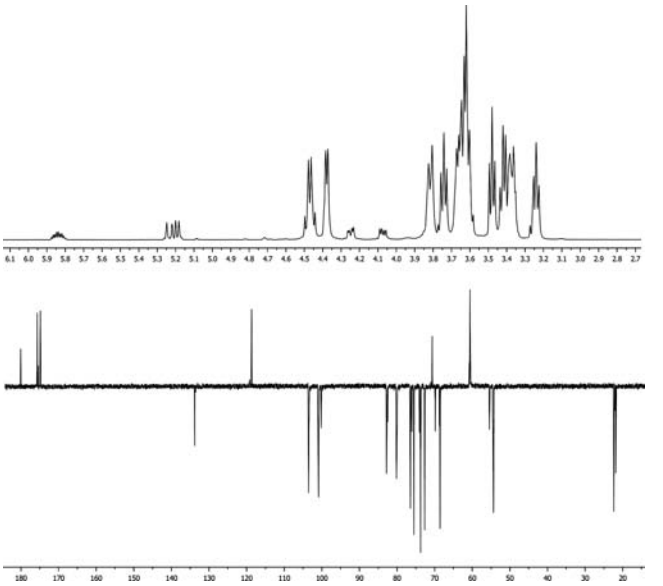

Figure 2. Fragments of the ^{1}H NMR (top) and ^{13}C NMR (bottom) spectra of pentadecamer **16**.

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

The authors declare no competing financial interest.