Straightforward Synthesis of Man₉, the relevant epitope of the High-Mannose Oligosaccharide

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The high mannose oligosaccharide (or its corresponding Man₉ epitope) is the most abundant structure presented in pathogen envelope glycoproteins. These glycans play a key role in the pathogenesis of several pathogens and also in the communication with the immune system. Understanding the mechanism of action of these glycans requires the access to pure and chemical well-defined structures in reasonable amounts. The synthesis of these complex branched oligosaccharides is not trivial and few syntheses are described in the literature with several synthetic and purification steps and low overall yields. In this work, we described a very efficient synthetic alternative to access to this relevant Man₉ epitope in a very straightforward way.

Introduction

The high-mannose oligosaccharide is a biologically relevant molecule that participates in quality control and intracellular transportation of glycoproteins.¹ Furthermore, N-linked high-mannose oligosaccharides are coating the surface of many pathogenic microorganisms such as viruses, bacteria, fungi and parasites, and are the target of immune system cells, mainly macrophages and dendritic cells (DCs).² The interactions of these oligosaccharides with animal lectins are of crucial importance for the efficient operation of the innate immune system. Examples include mannose-binding lectins (MBL), dendritic cells specific ICAM-3 grabbing non-integrin (DC-SIGN), Langerin, defensins and macrophages mannose receptors (MR).³ In fact, in some particular cases, the glycan structures on pathogen glycoproteins present in viral envelopes or bacterial cell walls help to escape recognition by the immune system and the subsequent elimination or neutralization of the pathogen.² In particular, DC-SIGN plays a main role in the pathogenesis of HIV-1.⁴ This virus targets DC-SIGN, but escapes degradation in lytic compartments, thus using DCs as a Trojan horse to migrate to lymphoid nodes, trans-infecting T-cells, invading the host

organism and causing the AIDS disease.⁴ In this context, DC-SIGN is currently considered as an interesting new therapeutic target for the design of anti-infective agents.⁵ Additionally, immunotherapy using DC-based vaccination is an approved approach for harnessing the potential of a patient's own immune system to eliminate tumor cells in metastatic hormone-refractory cancer.⁶ Therefore, targeting these receptors is becoming an efficient strategy to improve the immunogenicity of antigens in DC-based anticancer immunotherapy, especially in pre-clinical animal models and in vitro DC antigen presentation and T-cell stimulation assays.⁷ A major challenge for vaccine design is targeting antigens to human DCs in vivo, facilitating the cross-presentation, and conditioning the microenvironment for Th1- and Th2-type effective T-cell immune responses.⁷ Information at molecular level concerning the mechanism by which this receptor operates is scarce, thus effective modulators of DC-SIGN are also required to clarify the different biological pathways in which this receptor is involved.

The main carbohydrate ligand recognized by DC-SIGN is the high-mannose glycan, (Man)₉(GlcNAc)₂, a branched oligosaccharide containing mannose with α 1,2-, α 1,3-, α 1,6-, and β 1,4-linkages, being the mannosyl nonasaccharide (Man₉) the main epitope to interact with this receptor. Multiple copies of this glycan are present in several pathogen glycoproteins, like in the gp120 envelope protein of HIV. The total synthesis of Man₉ or (Man)₉(GlcNAc)₂ and other complex mannose oligosaccharides has been explored;⁸ however, the complexity of this kind of branched glycan structures prevent the accessibility to large amounts required to address biological studies. Previously described approaches to synthesize high-mannose type oligosaccharides involve many reaction steps with moderate to low yields in many cases, including the classical and well-established protection-deprotection pathway to prepare oligosaccharides, with several purifications of the intermediate products. Also, once the Man₉ or (Man)₉(GlcNAc)₂ skeleton is synthesized, the very low yield of the final

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In this synthetic approach, benzoyl esters have been selected as protecting groups making the synthesis compatible with the use of terminal azido, alkyne, alkene or aldehyde functionalized spacers, of great interest for the preparation of biologically relevant glycoconjugates or multivalent systems using click chemistry reactions.¹¹ The use of “disarmed” thioglycosyl donors in complex oligosaccharides synthesis is unusual, and no many examples are found in the literature. We also demonstrated that the use of these disarmed thioglycosyl donors is as adequate as trichloroacetimidate or fluoro “armed” donors.

Initially, the synthesis of the acceptor **5**, the most challenging fragment of this strategy due to the β linkage, was afforded. This synthesis was performed improving the β -mannosylation methodology using 2-*O*-propargyl ethers described by Crich and col.,¹² starting from the S-Tolyl mannoside **2** and reducing the number of purifications to only three silica gel chromatographies. (Scheme 2a) The synthesis started with the protection of C4 and C6 hydroxyl groups as a benzylidene acetal, following by regioselective *p*-methoxybenzoylation of the C3 hydroxyl groups using Bu₂SnO. Finally, protection of the C2 hydroxyl group with allyl bromide and NaH furnished fully protected S-Tolyl mannoside **3** in seven hours, after only one purification step and with 75% overall yield. Then, β -glycosylation of **3** with 2-bromoethanol by activation with 1-Benzenesulfinylpiperidine (BSP), Tf₂O and 2,4,6-Tri-*tert*-butylpyrimidine (TTBP) gave exclusively the β -mannoside **4**, as a single anomer, in 78% yield. (Scheme 2a)

Scheme 1. Retrosynthetic analysis for the Man₉ (1).

The β -stereochemistry of the anomeric center of compound **4** was unambiguously confirmed by the magnitude of the $J_{C-1,H-1}$ coupling constant ($J_{CH} = 155.4$ Hz) measured by means of a coupled HSQC NMR experiment. A similar technique was used to verify the stereochemistries of all newly created linkage.¹³ The corresponding values for all these coupling constants are reported in the Supplementary Information.

At this stage, we considered that once the propargyl group had done its function to achieve the β -linkage; it should be removed and substituted by a benzoyl group. In this way, we avoid the removal of the C2 propargyl group in the last steps of the synthesis that could compromise the overall yield and the final compound will have all the hydroxyl groups protected as benzoyl esters which should facilitate the final deprotection step to obtain the unprotected Man₉. To achieve this goal, we firstly addressed the substitution of the bromo by an azido group using a large excess of NaN₃. Then, propargyl ether was removed by isomerization to the corresponding allene with potassium *tert*-butoxide in THF and subsequent cleavage with catalytic osmium tetroxide (OsO₄) and *N*-methyl-morpholine *N*-oxide (NMNO). Benzoylation of the C2 hydroxyl group with benzoic anhydride (Bz₂O), Et₃N and DMAP following by removal of C3 *p*-methoxybenzyl ether using DDQ gave β -mannoside **5**. The synthetic strategy chosen to achieve the preparation of acceptor **5** allows a sequential synthesis of the five steps from **4** with only a final silica gel purification to afford this acceptor **5** in 74% yield. (Scheme 2a) We have performed also the preparation of this compound **5** isolating and characterizing every intermediate. (See SI)

The acceptor **6** was synthesized in only three synthetic steps following a procedure described in the literature.¹⁴ However, we observed that all single steps of the procedure would be performed in a consecutive way with only a final purification step. Starting from **2**, chemoselective protection of the C3 and C6 hydroxyl groups as silyl ether was performed using a slight excess of TBDMSCl and imidazole at 0°C to avoid the formation of mixtures of 4,6- and 2,6-O-di-TBDMS.¹⁴ Then, benzoylation of C2 and C4 free hydroxyl groups using an excess of Bz₂O, Et₃N and DMAP and, finally, the selective removal of silyl groups

using HF-Py complex gave the desired acceptor **6** with 70% overall yield after a final silica gel chromatography. (Scheme 2b)

Table 1. The reaction conditions used for reactivity-based one-pot self-condensation of **7**.

	TfOH (eq.)	NIS (eq.)	8 (%)	9 (%)
1	0.1	0.7	68	2
2	0.2	0.7	36	22
3	0.2	0.65	44	24
4	0.2	0.6	54	15

Once the **5** and **6** acceptors were prepared, the synthesis of Man₉ was afforded following the described approach in Scheme 1. The strategy required the synthesis of the disaccharide **13** and the trisaccharide **15**. Applying the “consecutive synthesis” concept described previously in our group⁹ together with the self-glycosylation approach introduced by Wong and col. in 2004,¹⁵ the simultaneous synthesis of both compounds have been addressed from monosaccharide **7**. The synthesis of 2-hydroxy free mannoside **7** was performed in five synthetic steps with only a silica gel chromatography with 65% yield.⁹ (Scheme 2c) Then, self-condensation of **7** using NIS and TfOH gave a less-reactive disaccharide **8**. This disaccharide served partially as acceptor for another monomer molecule to give the trisaccharide **9**. For this reason, compounds **8** and **9** were simultaneously obtained in one pot. Both compounds were converted in the fully protected disaccharide **13** and trisaccharide **15** by benzoylation with Bz₂O. The self-glycosylation reaction worked properly using benzoyl ester as protecting groups. Entry 3 in Table 1 shows the optimal conditions for the self-glycosylation to obtain a 2:1 ratio of disaccharides and trisaccharides. This ratio is the most convenient one for our approach because the disaccharide **13** was used, after conversion of the STol group to a trichloroacetimidate, in a 2+1 glycosylation of the acceptor **6** while the trisaccharide **15** is involved in a 1+1 glycosylation of the acceptor **5**. Both the consecutive synthesis of S-Tolyl mannoside **7** and the self-glycosylation were performed in multigram scale with excellent yields, including only two silica gel chromatographies and less than 48 hours. (Scheme 3)

Scheme 2. Consecutive synthesis of acceptors **5** and **6** and the donor/acceptor **7**. Scheme 3. The two approaches to synthesize the disaccharide **13** and the trisaccharide **15** from the S-Tol mannose derivative **7** and from baker's yeast acetolysis.

The second approach to obtain the disaccharide **13** and the trisaccharide **15** is based on a semisynthetic strategy using as starting material the very cheap and easy accessible dry baker's yeast found in supermarkets. Baker's yeast is the common name for the strains of yeast frequently used as a leavening agent in baking bread and bakery products. Baker's yeast is of the species *Saccharomyces cerevisiae*,¹⁶ its cell wall is covered by a dense layer of mannan, a mannose polysaccharide composed by an $\alpha(1-6)$ linked backbone and $\alpha(1-2)$ and $\alpha(1-3)$ linked branches. Szurmay and col.¹⁰ described the acetolysis of baker's yeast with Ac_2O and H_2SO_4 to obtain per-acetylated mannan oligosaccharides. Following this procedure, we obtained the corresponding pure peracetylated $\alpha(1,2)$ mannose disaccharide **10** (1.5 grams) and trisaccharide **11** (1.2 grams) directly from dry baker's yeast (50 grams) after purification by silica gel chromatography in only 10 hours. Acetolysis entails an enormous saving of time, reduces the reactions and purification steps, and decrease the economic impact in comparison with the stepwise synthesis. After

purification, disaccharide **10** and trisaccharide **11** were converted to the corresponding S-Tolyl derivatives **12** and **14** by direct glycosylation with 4-methylphenylthiol using $\text{BF}_3\text{-OEt}_2$ as promoter. Finally, interconversion of the acetyl groups by benzoyls provided the disaccharide **13** and the trisaccharide **15** with excellent yields. (Scheme 3)

With the four building blocks required in hand, the assembly of larger structures was addressed. We first focused on the preparation of the pentasaccharide **17**. The S-Tolyl leaving group of disaccharide **13** was converted to the corresponding trichloroacetimidate **16** by selective removal of S-Tolyl group with NBS and water and subsequent reaction of the C1 hydroxyl group with trichloroacetonitrile and DBU generating the corresponding trichloroacetimidate derivative in high yield. Then, 2+1 glycosylation of acceptor **6** with donor **16** yielded pentasaccharide **17** in a single step in 90% yield. (Scheme 4)

The glycosylation of the 3-OH of the acceptor **5** with the S-Tolyl donor **15** using NIS and TfOH gave the tetrasaccharide **18** bearing the benzylidene group spanning C4 and C6 with 91% yield. The acetal was removed in acid media with *p*-TsOH in acetonitrile affording the tetrasaccharide **19** in high yield. Finally, nonasaccharide **20** was assembled efficiently through the glycosylation of tetrasaccharide acceptor **19** with the pentasaccharide donor **17**. The regioselectivity observed for the hydroxyl group at position C6 in this reaction has been previously described in the literature^[17] and is due to the higher reactivity of the C6 primary hydroxyl group with regard to the secondary hydroxyl group at C4. The absence of steric hindrance group at the C4 position of the tetrasaccharide made more accessible the C6 hydroxyl group increasing the yield of the final glycosylation step to be almost quantitative (98%). The used of the thioglycosyl “disarmed” donor in this glycosylation step is the first example described in the literature. The previous synthetic approaches used in the high-mannose oligosaccharide synthesis always include the substitution of acyl by benzyl groups and the modification of the -STol or -SPh by other leaving groups as trichloroacetimidate or fluoride. The final step to achieve the target molecule Man₉ was the global deprotection of nonasaccharide **20**. This deprotection was performed in only one synthetic step using NaOMe in methanol following by aqueous NaOH solution to obtain the desired unprotected nonasaccharide **1** (Man₉) in quantitative yield. (Scheme 4)

Conclusions

In summary, we have completed a convergent, high yielded, fast and large amount accessible synthesis of the high-mannose nonasaccharide Man₉ (**1**) from four key intermediates: monosaccharide acceptors **5** and **6**, the disaccharide **13** and the

trisaccharide **15**. These latter ones have been prepared easily following two different strategies: “consecutive synthesis” and baker’s yeast acetolysis semisynthetic approach. The synthesis described in this work permits the access to high mannose oligosaccharides minimizing the time spent to perform the synthesis, reducing the purification steps and improving a lot the overall yield respect to the best procedure described in the literature. In the case of baker’s yeast approach, the complex building blocks required in the synthesis (di- and trisaccharides, **13** and **15**, respectively) were obtained in only 10 hours in multigrams scale starting from a very cheap and accessible natural source. We considered that using this strategy, now it is possible to access rapidly and in large amounts to the relevant Man₉ epitope, a very interesting ligand for DC-SIGN. Moreover, this strategy based on consecutive approaches allows an easy access to other linear or branched mannosyl derivatives of interest. On the basis of this development, and taking the advantage of the terminal azido group present in the spacer at the reducing end of this oligosaccharide, we are currently preparing Man₉ glycoconjugates via click chemistry using compound **1**. These new glycomimetics will be tested as anti-infective agents or in combination with immunogenic peptides to be used in immunotherapy.

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