Synthetic Neoglycoconjugates of Hepta- and Nonamannoside Ligands for Eliciting Oligomannose-Specific HIV-1-Neutralizing Antibodies

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Dedicated to Joachim Thiem at the occasion of his 80th birthday

Oligomannosetype glycans on the spike protein of HIV-1 constitute relevant epitopes to elicit broadly neutralizing antibodies (bnAbs). Herein we describe an improved synthesis of α- and β-linked hepta- and nonamannosyl ligands that were subsequently converted into BSA and CRM197 neoglycoconjugates. We assembled the ligands from anomic 3-azidopropyl spacer glycoses from select 3-O-protected thiocresyl mannoside donors. Chain extensions were achieved using [4 + 3] or [4 + 5] block synthesis of thiocresyl and trichloroacetimidate glycosyl donors. Subsequent global deprotection generated the 3-aminopropyl oligosaccharide ligands. ELISA binding data obtained with the β-anomeric hepta- and nonamannosyl conjugates with a selection of HIV-1 bnAbs showed comparable binding of both mannosyl ligands by Fab fragments yet lesser binding of the nonasaccharide conjugate by the corresponding IgG antibodies. These results support previous observations that a complete Man9 structure might not be the preferred antigenic binding motif for some oligomannose-specific antibodies, and have implications for glycoside designs to elicit oligomannosetargeted HIV-1-neutralizing antibodies.

Introduction

Developing an effective vaccine against HIV-1 has not been successful despite many years of intense efforts.[1,2] The HIV-1 envelope spike (Env) harbors a dense array of glycans that may serve as targets for vaccine development, given their potential to generate broadly neutralizing antibodies (bnAbs).[3] Among the heavily glycosylated patches of gp120, oligomannose-type glycan clusters constitute neo-epitopes suitable for eliciting bnAbs, as exemplified by the prototype PGT125-131 family of antibodies.[4,5] Previous attempts to produce oligomannose-specific bnAbs used glycoconjugates with dense oligomannosyl clusters, but these attempts have only modestly been successful.[6] The reasons for the lack of success are not clear. One hypothesis, reviewed elsewhere,[1] is that, due to the similarity of synthetic oligomannosides to self-antigens, tolerance mechanisms may prevent the development of proper cross-reactive B cells. Another hypothesis, suggested by recent findings, is that serum mannosidases, first reported some time ago,[6] rapidly degrade oligomannose-containing ligands and thus prevent B cell recognition.[7,8] Preferential B cell recognition of only select parts of the neoglycosides, rather than the whole moiety, cannot be ruled out entirely either.[1]

We have previously shown that a bacterial oligomannoside mimic[2] elicits modest neutralizing activity, with binding specificities comparable to those of the PGT125-131 bnAb family members.[1,4] The lead mimetic comprised the so-called “D1” and “D3” arm of the Man, epitope and was equipped with a β-linked 3-propylamino aglycon for conjugation to a protein carrier (e.g., bovine serum albumin or the diphtheria-derived CRM197).[9] No HIV peptide sequence is included because of reports showing that glycopeptides elicit nearly exclusively anti-peptide, rather than anti-glycan antibodies.[10] During the previous synthesis, the β-anomeric spacer derivative 2 had been prepared from the commonly used thioglycoside pentamannosyl donor 1, albeit as the minor anomer (α/β ratio 2.5:1) (Scheme 1).

We set out to improve the overall yield for the preparation of the lead β-heptamannoside antigen, based on an early introduction of the spacer unit and to synthesize additional building blocks to access the complete Man9, nonasaccharide. The α-configured heptamannoside had successfully been used in a crystallographic studies with the PGT128 bnAb and since the corresponding BSA conjugates were also bound – albeit with lower avidity- to PGT128 and the 2G12 Ab, we include the corresponding BSA conjugates were also bound – albeit with lower avidity- to PGT128 and the 2G12 Ab, we include the synthesis of the α-nonasaccharide.[11,12] We also report on the preparation of the corresponding neoglycopeptides as bovine
serum albumin (BSA) and CRM197 conjugates and ELISA binding studies with select members of the PGT125-131 bnAb family.

Results and Discussion

To improve the overall yield of the β-configured ligands, we first investigated the challenging synthesis of the 1,2-cis connected spacer aglycon at the monosaccharide stage. We based our investigation on torsionally disarmed 4,6-O-benzylidene mannopranosyl thioglycosides, as investigated thoroughly by Crich and others. Additional approaches towards β-selective mannosides have been reported. Notable examples are intramolecular (IAD) and hydrogen-bond-mediated aglycon delivery, C-2 inversion of β-glucopyranosides, anhydro derivatives, and anomeric alkylation. The 4,6-O-benzylidene protocol provides a versatile intermediate for the late introduction of the 1-6 mannosyl arm and several options for introducing orthogonal protecting groups. Furthermore, installing ether-type protecting groups with low steric impact at the 2-O- and 3-O-positions has been beneficial for exquisite β-selective properties in the respective glycosyl donors. Since we aimed for a robust and scalable method to efficiently synthesize anomic spacer mannosides, a series of thiocresyl 4,6-O-benzylidene mannosides, protected differently at positions 2 and 3, were prepared. This alternate method also optimizes the protecting group strategy to synthesize both anomic glycosides efficiently. Starting from the known S-tolyl mannoside 3, a 3-O-(2-naphthalenylmethyl) group was installed as described, followed by alkylation of 5 with propargyl bromide in the presence of NaH in DMF to afford 6a in 95% yield (Scheme 2). The 3-O-(1-naphthylpropargyl) derivative 6c was synthesized from 3 via the known 2-O-benzyl thioglycoside 4 followed by subsequent alkylation in 95% yield. Donors 6b, 6d, 6e, and 6f were prepared as described. Sulfoxide 6g was obtained by oxidizing thiogly-

Scheme 1. Previously used route to introduce the β-anomeric spacer group.

Scheme 2. Synthesis of the anomic spacer glycoside derivatives 8a–8f.
The anomeric configuration of the spacer glycosides was verified by measuring the heteronuclear J_C1,H1 coupling constants.\textsuperscript{[19]}

We synthesized the oligosaccharides once selective access towards both anemic spacer mannosides was established. Assembly was first elaborated for the α-series starting from 8f. The 3-O-benzoyl ester was removed under Zemplén conditions to give the glycosyl acceptor 9 in 94% yield (Scheme 3). For assembly of the D1 arm comprising the α-(1→3)-linkage to the reducing mannose unit, the trichloroacetimidate glycosylation approach was selected.\textsuperscript{[40]} Coupling of the previously described\textsuperscript{[41]} trisaccharide donor 10 to the acceptor 9 thus proceeded smoothly with the help of TMSOTf within 1 h at room temperature. It afforded the α-(1→3)-linked tetrasaccharide 11 in excellent yield (91%). Regioselective reductive opening of the 4,6-O-benzylidene acetal using triethylsilane and PhBCl\(_2\) at low temperature produced the primary alcohol 12 in 96% yield.

The previously described\textsuperscript{[42]} primary alcohol 13 was glycosylated with the disaccharide trichloroacetimidate donor 14\textsuperscript{[43]} to obtain the linear trimannosyl donor 15. TMSOTf was used to promote glycosylation, thus producing the α-(1→6)-extended thioglycoside donor 15 in 85% isolated yield (Scheme 4).\textsuperscript{[44]} The 3-O-Nap derivative 13 was subjected to DDQ-oxidation to generate diol 16 in an 86% yield to access the branched pentasaccharide donor 17. Simultaneous introduction of the D2 and D3 arms was then achieved by glycosylation of this diol acceptor with the disaccharide donor 14.\textsuperscript{[45]} Assignment of the α-anomeric configurations was supported by the heteronuclear coupling constants J_C1,H1. Next, the protected heptasaccharide 18 was synthesized by glycosylation of the primary alcohol 12 with the trisaccharide thioglycoside donor 15. NIS/triflic acid promoted the reaction, and an 82% yield was achieved. Due to the 2-O-participating benzyl group in donor 15, the reaction proceeded with full stereocontrol, leading to the α-(1→6)-linked heptasaccharide 18. Global deprotection was carried out by transesterification of the acyl groups and hydrogenolysis of the benzyl and Nap ether groups, as described previously.\textsuperscript{[13]} Deprotection was done under microflow conditions and with concomitant reduction of the terminal azide group. Final purification by chromatography on Sephadex LH-20 gave the previously reported α-heptasaccharide 19 as an acetate salt with a 95% yield.\textsuperscript{[13]} NMR data were in agreement with an authentic sample.

Proceeding towards the nonamannoside derivative 21, we followed a straightforward strategy using a [4 + 5] block-wise assembly.\textsuperscript{[46]} Indeed, coupling the branched pentasaccharide
donor 17 to the tetrasaccharide glycosyl acceptor 12 promoted by NIS/TfOH worked equally well and afforded the protected nonasaccharide derivative 20 in 73% isolated yield. Deprotection as described for the heptasaccharide 19 furnished the nonasaccharide 21 in 81% yield (Scheme 5). NMR analysis of 21 based on COSY, TOCSY, HSQC, and HMBC measurements confirmed the structural assignments. Signals for the anomeric carbons of the three distal manno.pyranosyl residues were observed as overlapping singlets between 102.3–102.2 ppm. The anomeric carbon signals of the two units engaged in the α-(1→3)-linkages at 100.75/100.77 ppm could also not be resolved, whereas the remaining anomeric signals were well separated and individually assigned (for full labeling of the nonasaccharide units see Supporting Information). Glycosylation shifts were observed for carbon 2 of four mannose residues as well as for the fully assigned signals for carbon 3 and carbon 6, respectively, for the four innermost mannose units.

Synthesis of hepta- and nonasaccharides containing the β-linked spacer group

The β-spacer monosaccharide derivatives had been obtained with high stereoselectivity, providing several options for generating the glycosyl acceptor 22. Removal of the 3-O-propargyl group of 8b using KOrBu/OsO₄ in the presence of NMMO worked well, but we did not pursue this at a larger scale due to toxicity concerns. Instead, DDQ-oxidation of the Nap and PMB groups of 8c, 8d, and 8e was carried out in good yields, allowing for isolation of the pure β-anomers when using the anemic mixtures of 8d and 8e, respectively (Scheme 6).

Following the conditions elaborated for the α-spacer derivative, glycosylation of 22 with the trichloroacetimidate donor 10 promoted by TMSOTf proceeded smoothly. This reaction gave the α-(1→3)-linked tetrasaccharide 23. Subsequent regioselective reductive opening of the 4,6-O-benzylidene group afforded the primary alcohol 24. This alcohol was ready for elongation with the trisaccharide and pentasaccharide building blocks 15 and 17. Coupling the thioglycoside donor 15 with 24 in the presence of NIS/TfOH was uneventful and provided the protected heptasaccharide 25 in 78% yield (Scheme 7). Deprotection of 25 and reduction of the terminal azide afforded the previously described[13] heptasaccharide ligand 26 in 78% yield. Similarly, the reaction of 24 with the pentasaccharide donor 17 led to the nonasaccharide derivative 27 in 80% yield followed by processing as described for 26 to afford the nonasaccharide target derivative 28 in 83% yield. The NMR data of 28 were in excellent agreement with the fully assigned signals of Man₉GlcNAc₂, except for the different reducing end mannosyl residue due to a different chemical environment at the reducing end of the oligomannose glycan.[45] The overall yield in the preparation of the β-spacer heptasaccharide 26 involving five steps calculated from the [3+1] coupling and the subsequent elongation of the tetrasacchar-
ide 23 is 42%. In our previous approach with a late-stage introduction of the spacer group, seven steps were needed that gave an overall yield (again based on the first [3 + 1] glycosylation) of 8.5%.

Synthesis of BSA and CRM\textsubscript{197} neoglycoconjugates and definition of their antigenicity

For consistency with previously made conjugates\textsuperscript{[13]} neoglycoconjugates were prepared with an intended loading of 4–6 ligands per protein using optimized reaction conditions (Scheme 8). First, the terminal amino group of the β-anomeric spacer glycosides 26 and 28 were activated by thio phosphogene treatment to obtain intermediate isothiocyanates\textsuperscript{[46]} This was followed by coupling to the ε-amino groups of lysine residues on bovine serum albumin (BSA) and the diphtheria-derived cross-reactive material CRM\textsubscript{197}, respectively. The resulting neoglycoconjugates 29–32 were separated from their oligosaccharide ligands by spin filtration and kept as aqueous solutions. MALDI-TOF analysis of the neoglycoconjugates indicated copy numbers of 8.6, 5.6, 3.4 and 1.9 ligands/protein for conjugates 29, 30, 31, and 32, respectively.

The antigenicity of the hepta- and nonamannosyl CRM\textsubscript{197} conjugates was determined by ELISA using Fab and IgG versions of the PGT125, PGT126, PGT128, and PGT130 bnAbs. The specificity of these antibodies is fairly well-defined\textsuperscript{[4,5,47]}...
average of 2.6 heptamannosyl ligands per CRM$_{197}$ for comparison (NIT211-6). As shown in Figure 1, all four Fabs bind greater to the heptasaccharide conjugate 30 (MAC093) than the previously made glycoconjugate. This difference is possibly due to the higher carbohydrate/protein ratio (5.6 ligands/protein) on the current heptasaccharide conjugate compared to the earlier glycoconjugate (2.6 ligands/protein). All four Fabs bound the nonasaccharide conjugate 32 (1.9 ligands/protein) with an apparent affinity similar to the NIT211-6 conjugate. The absence of binding preference by the Fabs for conjugated hepta- or nonamannosyl ligands confirms that these ligands are antigenically equivalent.

In contrast to the Fabs, a distinct preference in binding for both heptasaccharide conjugates (MAC093, NIT211-6) relative to the nonasaccharide conjugate (MAC095) was observed with IgG versions of the antibodies (Figure 2). One possible explanation is that the ligands on the nonasaccharide are spaced too far apart to allow bivalent IgG binding; follow-up studies with nonasaccharide conjugates loaded at higher density will help to address this possibility. Another possibility is that the D2 arm in

![Scheme 8. Synthesis of the hepta- and nonamannosyl BSA and CRM$_{197}$ conjugates 29–32.](image)

**Figure 1.** Binding of Fab fragments of bnAb PGT125, PGT126, PGT128, and PGT130 to the β-heptamannosyl conjugates 30 (MAC093 and NIT211-6) as well as the nonamannosyl conjugate 32 (MAC095). The two β-heptamannosyl and nonamannosyl conjugates were coated as solid-phase antigens onto ELISA plates at 76, 81, and 81 nM concentration.
the nonasaccharide, absent in the heptasaccharide, hinders bivalent IgG engagement in the context of the neoglycoconjugate. This possibility agrees with observations from the first studies on the glycan specificity of these PGT antibodies, which showed that they bind poorly to Man₉ connected to the relatively rigid chitobiose core;[16] the antibodies bound Man₉ only when the ligand was attached to a flexible linker (–(CH₂)₆–NH–). If correct, then the relatively poor binding of the IgGs to the nonasaccharide conjugate here suggests that the isothiouronium linker does not afford sufficient flexibility to allow antibody binding to Man₉. The observation[16] that the PGT IgGs bind relatively strongly to chitobiose core-conjugated Man₉, which has a shortened D₂ arm, supports the influential role of the D₂ arm.

Conclusion

We successfully prepared anomerically pure 3-azidopropyl mannosides from 4,6-O-benzylidene protected thiocresyl glycosyl donors under Crich conditions. High yields and excellent stereoselectivities were achieved. Depending on the availability of an electron- withdrawing ester group or electron-donating arylalkyl or propargyl group at O-3, the glycosylation reaction with 3-azido-1-propanol afforded the α-glycoside or the β-linked product, respectively. Selective removal of the orthogonal 3-O-protecting group followed by coupling with an α-(1→2)-linked trimannosyl trichloroacetimidate donor gave the respective anomerically tetrasaccharides in high yields. Reductive opening of the benzylidene acetal provided the corresponding primary alcohols for ensuing [4+3] and [4+5] block-wise elongation with thioglycoside donors. Global deprotection afforded the benzylidene acetal provided the corresponding primary alcohols for ensuing [4+3] and [4+5] block-wise elongation with thioglycoside donors. Global deprotection afforded the corresponding primary alcohols for ensuing [4+3] and [4+5] block-wise elongation with thioglycoside donors. Global deprotection afforded the corresponding primary alcohols for ensuing [4+3] and [4+5] block-wise elongation with thioglycoside donors. Global deprotection afforded the corresponding primary alcohols for ensuing [4+3] and [4+5] block-wise elongation with thioglycoside donors. Global deprotection afforded the corresponding primary alcohols for ensuing [4+3] and [4+5] block-wise elongation with thioglycoside donors.

Experimental Section

General methods: All purchased chemicals were used without further purification unless stated otherwise. Solvents were dried over activated 4 Å (CH₂Cl₂, N,N-dimethylformamide, pyridine) molecular sieves. Dry MeOH (Merck) was purchased. Cation exchange resin DOWEX 50 H⁺⁺ was regenerated by washing with HCl (3 M), water, and dry MeOH. Aqueous solutions of salts were saturated unless stated otherwise. The concentration of organic solutions was performed under reduced pressure < 40 °C. Optical rotations were measured with an Anton Paar MCP100 polarimeter. [α]D values are given in units of 10⁻¹°deg cm g⁻¹. Thin-layer chromatography was performed on Merck precoated plates: 5 × 10 cm, layer thickness 0.25 mm, Silica Gel 60F₂₅₄ alternatively on HPTLC plates with 2.5 cm concentration zone (Merck). Spots were detected by dipping reagent (anisaldehyde-H₂SO₄). Silica gel (0.040–0.063 mm) was used for column chromatography. HP-column chromatography was performed on pre-packed columns (YMC-Pack SIL-06, 0.005 mm, 250 × 10 mm and 250 × 20 mm). NMR spectra were recorded on a Bruker Avance III 600 instrument (600.2 MHz for 1H spectra were referenced to δ = 0 using the TMS signal for solutions in CDCl₃, and 13C spectra were referenced to 77.00 (CDCl₃), 54.00 (CD₂Cl₂) and 67.40 (D₂O, external calibration to 1,4-dioxane) ppm. Assignments were based on COSY, HSQC, HMBC, and TOCSY spectra. ESI-MS data were obtained on a Waters Micromass Q-TOF Ultima Global instrument. MALDI data were obtained on a Bruker Autoflex MALDI TOF/TOF instrument using 2,5-dihydroxy acetophenone as the matrix. Synthetic procedures are described in the Supporting Information.

Neoglycoconjugate 29: A solution of thiophosgene (8 µL, 0.11 mmol) in CHCl₃ (1.5 mL) was added to a solution of 26 (2.0 mg,
ELISA: ELISAs were performed as described previously. Briefly, neoglycoconjugates were absorbed onto 96-well polystyrene ELISA plates (Corning) at 5 μg/mL in PBS overnight at 4 °C. After washing, the plates were blocked with PBS supplemented with 3% (w/v) BSA (PBS-B). Antibodies serially diluted in PBS supplemented with 1% (w/v) and 0.02% (v/v) Tween-20 (PBS-BT) were then added. After washing, bound Fab was detected with biotin anti-IgG-CH1 conjugate (Thermo Scientific) and IgG with peroxidase-conjugated antibodies (Thermo Scientific) and IgG with peroxidase-conjugated antibodies (Thermo Scientific) and IgG with peroxidase-conjugated antibodies (Thermo Scientific) and IgG with peroxidase-conjugated antibodies (Thermo Scientific). The data that support the findings of this study are available from the corresponding author upon reasonable request.

Data Availability Statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflict of Interest
The authors declare no conflict of interest.

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