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β-1,2-Oligomannan phosphorylase-mediated synthesis of potential oligosaccharide vaccine candidates

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ABSTRACT

β-(1,2)-Mannan antigens incorporated into vaccines candidates for immunization studies, showed that antibodies raised against β-(1,2)-mannotriose antigens can protect against disseminated candidiasis.

Until recently, β-(1,2)- mannans could only be obtained by isolation from microbial cultures, or by lengthy synthetic strategies involving protecting group manipulation. The discovery of two β-(1,2)-mannoside phosphorylases, Teth514_1788 and Teth514_1789, allowed efficient access to these compounds. In this work, Teth514_1788 was utilised to generate β-(1,2)-mannan antigens, tri- and tetra-saccharides, decorated with a conjugation tether at the reducing end, suitable to be incorporated on a carrier *en-route* to novel vaccine candidates, illustrated here by conjugation of the trisaccharide to BSA.

1. Introduction

β-D-Mannopyranose is found as a component of a variety of natural oligo and polysaccharides that play essential roles in pathological and biological processes. For example, mannose moieties exists widely in protein *N*-glycans [1], plant cell walls, yeast and various fungi [2]. β-(1,2)-Mannooligosaccharides have shown great potential as vaccine components against pathogenic *Candida* [3]. Immunization studies in mouse models showed that synthetic β-(1,2)-mannan antigens could be incorporated into vaccines as an effective antigen and particularly the β-(1,2)-mannotriose has been identified as the optimal binder for a protective monoclonal antibody generated against disseminated candidiasis [4]. Glycoside phosphorylases are carbohydrate-active enzymes (CAZymes) (URL: http://www.cazy.org/) [5] that have been used as catalysts for glycoside synthesis including the production of α- and β-1,4-linked glucans [6,7]. Previously, we investigated the ability of wild-type cellodextrin phosphorylase (CDP: β-1,4-glucan linkage dependent) and laminaridextrin phosphorylase (Pro_7066: β-1,3-glucan linkage dependant) to tolerate a range of both natural and unnatural sugar 1-phosphate donors and glucan acceptor substrates [8,9].

Until recently, β-(1,2)-mannooligosaccharides could be obtained by isolation from microbial cultures [10], or multistep chemical synthesis [11] which requires extensive protecting group manipulation. The β-(1,2)-mannosyl linkage is challenging to achieve with either chemical or enzymatic synthesis [12]. Two β-(1,2)-oligomannan phosphorylases, Teth514_1788 and Teth514_1789, from *Thermoanaerobacter* sp. X-514, catalyse the synthesis of linkage specific β-(1,2)-oligomannan using β-(1,2)-mannobiose or D-mannose as the optimal acceptors, in the presence of the donor α-D-mannose 1-phosphate (Man-1-P) [13]. The kinetic studies for the biosynthetic assembly of β-(1,2)-oligomannan indicate that Teth514_1789 mainly catalyses the synthesis of β-(1,2)-mannobiose [14] while Teth514_1788 can produce oligomannan containing a higher degree of polymerisation (DP $\geq$ 3).

Herein, we report the application of Teth514_1788 to generate β-(1,2)-mannan antigens, specifically mannotriose and mannotetraose, and a demonstrative conjugation of mannotriose to BSA. The donor and acceptor specificity of Teth514_1788 was also investigated. The present study offers a robust strategy to access β-(1,2)-mannobioside antigens

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that can be incorporated into targeted vaccination therapies.

2. Results and discussion

2.1. Synthesis of β-(1,2)-mannooligosaccharides

Enzymatic synthesis of oligosaccharides allows fast, linkage specific generation of complex carbohydrate structures with great advantages over chemical synthesis [1]. Previously, the one-pot two-enzyme synthesis of β-(1,2)-mannotriose using *in situ*-generated Man-1-P and catalysed by β-(1,2)-mannobiose phosphorylase from *Thermoanaerobacter* sp. X-514 has been reported with a 57% yield (Liu et al., 2021). To demonstrate the suitability of β-(1,2)-mannoside phosphorylase Teth514_1788 as a catalyst for mannooligosaccharide synthesis, we first aimed at preparative-scale synthesis of β-(1,2)-mannotriose and β-(1,2)-mannotetraose. A commonly used conjugation strategy is reductive amination, that allows conjugation to carrier(s) without further modification of the oligosaccharide, however losing the heterocycle conformation of the carbohydrate at the reducing end. To retain the intact ring structure for the three mannose moieties we therefore needed to access either the DP 3 mannan with an anomeric handle for conjugation, or the longer DP 4 material.

In these syntheses, Man-1-P and D-mannose were used as the donor and the acceptor substrates, respectively, and the recombinant Teth514_1788 as the catalyst. Teth514_1788 was expressed in *E. coli* and purification of the protein was carried out as described in the experimental section (Chiku et al., 2014). Following the published protocol for the reaction conditions (Chiku et al., 2014), preparative synthesis of β-(1,2)-mannooligosaccharides was carried out in the presence of β-(1,2)-oligomannan phosphorylase Teth514_1788 (10 mg mL^{-1}) which catalysed the reaction of D-mannose (2.0 mM) with 4-fold excess of Man-1-P (8.0 mM) in sodium acetate buffer (100 mM, pH 5.0) for 6 h at 37 °C to generate the target oligosaccharides (Scheme 1), as confirmed by TLC (Fig. 1A). Teth514 _1788 was removed from the reaction mixture by heating to precipitate the protein and filtered through a 0.22 μm filter. The resulting solution was analysed by high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD), which indicated the formation of oligosaccharides in the DP2-DP8 range (Fig. 1B). The peaks appearing after DP 8 in the HPAEC profile of the reaction mixture have been rationalised as M-1-P and a series of oligomers originating from Man-1-P acting as both donor and acceptor and forming β-1,2-mannooligosaccharides capped with phosphate at the reducing end. While this hypothesis was not followed up directly, the likely tolerance of Teth514_1788 to a mannose glycoside acceptor was later explored (*vide infra*).

To remove buffer salts, the excess of Man-1-P and the oligomers bearing Man-1-P terminus, the reaction mixture was passed through an activated charcoal/Celite (50:50) column first eluted with water. Oligosaccharides were then eluted from the column with 95% ethanol and concentrated to dryness to yield 45 mg of the products mixture. HPAEC-PAD analysis of the mixture confirmed the presence of mannooligosaccharides with DP2 – DP8 and a small number of minor components eluted after the DP8 specie (Fig. 1C).

The hypothesised degree of polymerisation of β-(1,2)-mannooligosaccharides, assigned based on the HPAEC-PAD chromatogram profile, were confirmed by MALDI-TOF mass spectrometry, which exhibited peaks with *m/z* consistent with the calculated masses for the sodium adducts of mannooligosaccharides with DP3 – DP12 (Figs. S2 and S3).

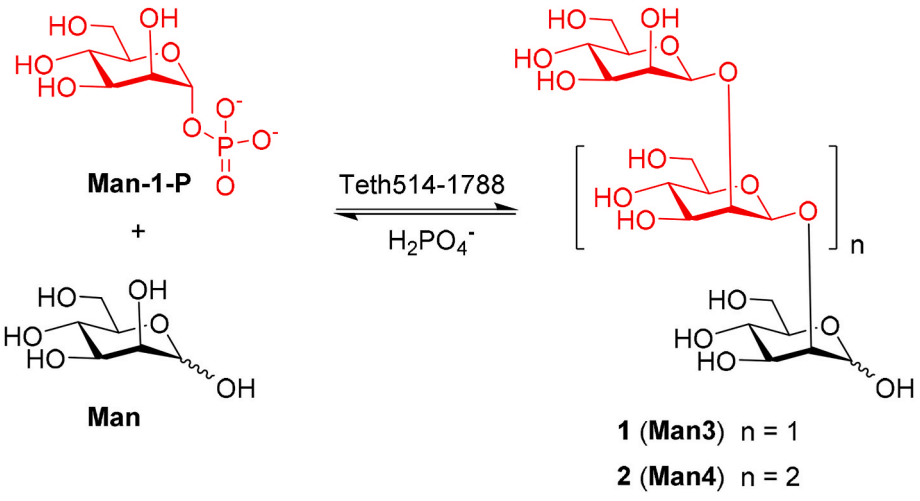
To demonstrate the feasibility of Teth514 _1788 catalysed formation of β-(1,2)-mannooligosaccharides as suitable antigens, we initially planned to synthesise β-(1,2)-mannotriose and β-(1,2)-mannotetraose starting from the mannose 1-phosphate (Man-1-P)/D-mannose donor/acceptor substrates. The reaction was carefully monitored and the outcome revealed that free mannose added as the acceptor substrate in the presence of an excess of donor substrate Man-1-P (4.0 equiv.) drove the reaction towards completion. Mannobiose was the main product, while β-(1,2)-mannotriose and β-(1,2)-mannotetraose species accumulate after mannobiose reached its maximum concentration. Therefore, reaction was monitored by TLC analysis and once β-(1,2)-mannotetraose was observed it was immediately terminated by boiling the sample at 95 °C for 10 min. Preparative synthesis of β-(1,2)-oligomannosides was carried out with β-(1,2)-oligomannan phosphorylase (10 mg mL^{-1}) catalysed reaction containing free mannose (2.0 mM) as the acceptor substrate in the presence of an excess of donor, Man-1-P (8.0 mM) in sodium acetate buffer (100 mM, pH 5.0) for 6 h at 37 °C to generate oligosaccharides, as confirmed by TLC (Fig. 1A).

2.2. β-(1,2)-mannotriose and β-(1,2)-mannotetraose

The molecular weight and structure of β-(1,2)-mannotriose (Man 3) and β-(1,2)-mannotetraose (Man 4) purified *via* gel permeation chromatography (Fig. 2) were confirmed by ESI mass spectrometry and NMR spectroscopy, respectively.

ESI-MS analysis of Man3 and Man4 revealed the presence of peaks at *m/z* 526.9, and *m/z* 688.8 respectively. These signals were consistent with *m/z* calculated for sodium adducts of those molecules: [**Man3**+Na]$^+$ at *m/z* 527.2 Da and [**Man4**+Na]$^+$ at *m/z* 689.2 (Fig. S2, Table S1).

^{1}H and ^{13}C NMR assignment for Man 3 and Man 4 were in agreement



Scheme 1. Synthesis of β-(1,2)-mannooligosaccharides with Man 2 to Man 12. Only compound **1** (Man3) and **2** (Man4) were purified.

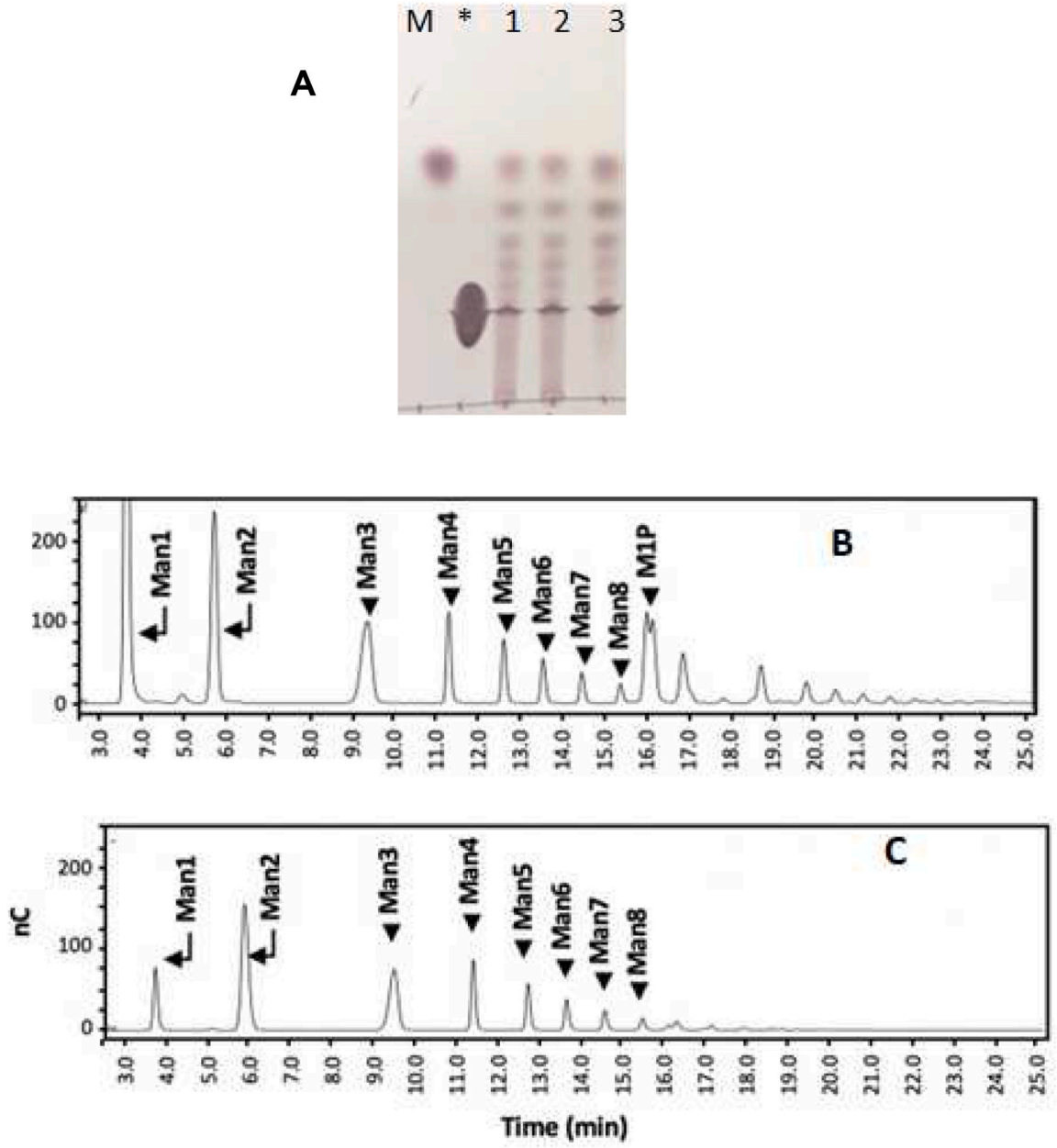


Fig. 1. A) Enzymatic synthesis of β-(1,2)-mannosides analysed by TLC. Solvent system: BuOH/EtOH/H_2O (10:8:7). M = mannose, * = Man-1-P, 1 = 10 min, 2 = 20 min, 3 = 6 h. TLC developed using orcinol spray and charring. HPAEC-PAD analysis of enzymatically synthesised β-(1,2)-mannooligosaccharides. Numbers indicate the DP for each peak. **B)** before purifying on charcoal/Celite (1:1) Chromatography column and **C)** After purification.

with data reported in the literature [10] confirming their structures as mannose oligosaccharides with β-(1,2) linkages, (Figs. S4 and S5).

2.3. Azide functionalisation of β-(1,2)-mannooligosaccharides

Introduction of an azide functional group at the anomeric position of both β-(1,2)-mannotriose and β-(1,2)-mannotetraose (Fig. 3) would allow either copper free or copper-catalysed click chemistry to be utilised for the conjugation to an alkyne. The installation of the azide was achieved on the anomeric position of the β-(1,2)-mannosides following *Shoda* methodology [15].

Man3-N_3 was isolated in 80% yield and NMR analysis showed a mixture of the expected anomeric β-azide with a small amount of α-azide. Further purification of this mixture was not pursued. Chemical shifts at 5.0 ppm in ^{1}H NMR and 86.9 ppm ^{13}C NMR spectra of Man3-N_3 were consistent with the presence of an anomeric β-azide. A smaller signal at 5.6 ppm in ^{1}H NMR spectra, correlating with 87.6 ppm in ^{13}C NMR spectra can be attributed to the anomeric α-azide. NMR analysis of Man4-N_3, which was produced in 87% yield, also confirmed the formation of the anomeric β-azide (with a small amount of α-azide present) as followed from ^{1}H NMR chemical shift at 5.0 ppm, which correlated with a^{13}C NMR chemical shift at 86.8 ppm. ESI-MS analysis supported the structure of Man3-N_3 and Man4-N_3 by the presence of $[M+Na]^+$ adducts with *m/z* at 551.9 and 714.0, respectively.

Considering the arduous separation of the anomeric azide mixture these compounds were not pursued further due to the risk of compromising the biological evaluation downstream. Instead, an approach based on an acceptor mannoside pre-derivatized at the anomeric position was investigated.

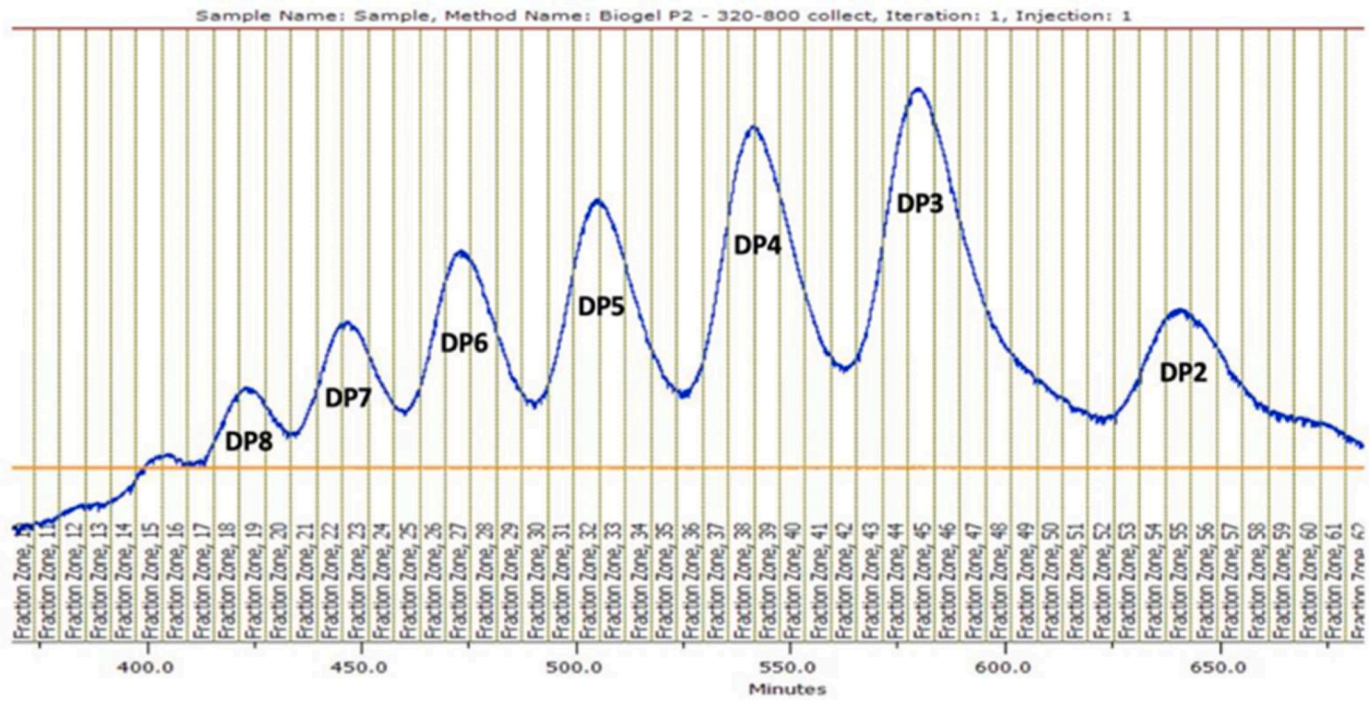


Fig. 2. Fractionation of a mixture of mannooligosaccharides with DP2 – DP8 Gel permeation chromatography (GPC) was performed using a Biogel P2 column (2.5 × 90 cm), 3 mL fractions were collected between 160 and 400 mL.

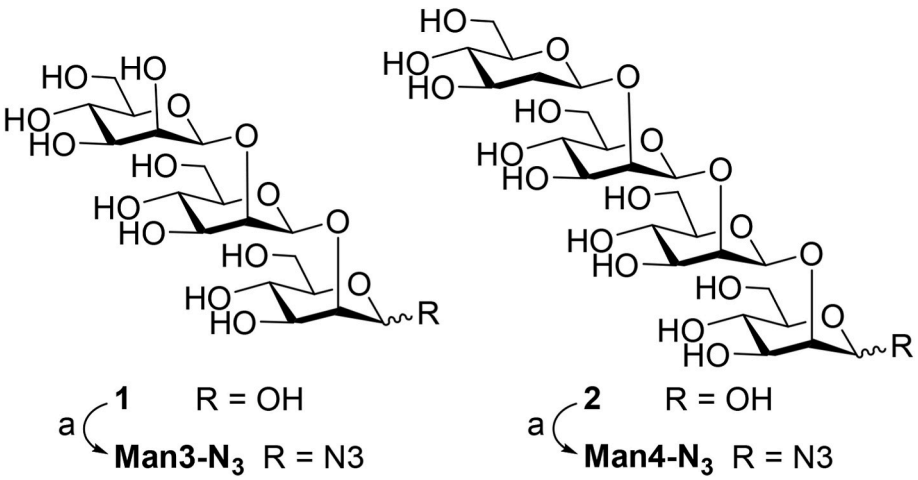
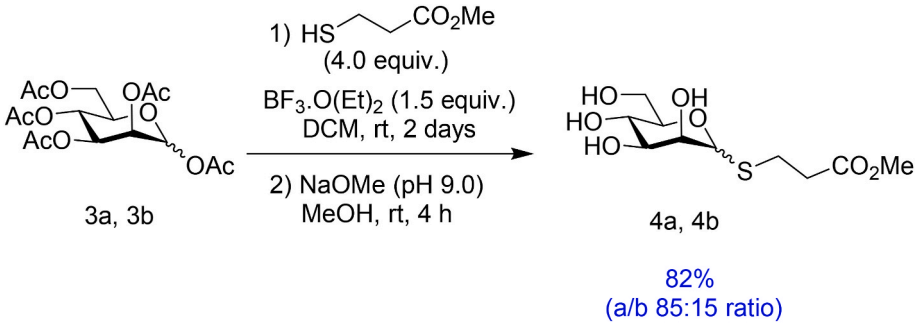


Fig. 3. Synthesis of azide-functionalized mannooligosaccharides: a) NaN_3 (2.5 M), 2-chloro-1,3-dimethylimidazolinium chloride (DMC) (5.0 eq.), *N*,*N*-diisopropylethylamine (DIPEA) (15.0 eq.), H_2O, 18 h, rt.



Scheme. 2. Synthesis of methyl 3-(α-D-mannopyranosylthio)propanoate **4a** and **4b**. Reagents and conditions: **a)** $HSCH_2CH_2CO_2Me$, $BF_3{\cdot}OEt_2$, DCM, rt, 2 days; **b)** NaOMe, dry MeOH, rt, 4 h.

2.4. Anomeric-functionalized β-(1,2)-mannooligosaccharides

2.4.1. Synthesis of methyl 3-(α-D-mannopyranosylthio)propanoate as a modified acceptor

The installation of thiopropanoate tether at the anomeric position of mannose can be performed starting from the readily available per-acetylated mannose and seemed a valid alternative strategy to access a versatile acceptor prior to the enzymatic synthesis of β-(1,2)-mannooligosaccharides. Reaction conditions to prepare methyl 3-(α-D-mannopyranosylthio)propanoate **4**, are reported in Scheme 2. Based on previously reported results, it was expected that anomeric configuration of the acceptor can be tolerated by the enzyme catalysing α-(1,2)-mannosylation. Compound **4a** was prepared by $BF_3{\cdot}OEt_2$-catalysed reaction of mannose penta-acetate **3** with methyl 3-thiopropanonate [16] which was then followed by deprotection using Zemplén conditions (Scheme 2). ^{1}H NMR analysis revealed that this procedure let to the formation of a small amount of β-anomer **4b** as well the expected α-anomer **4a** (85:15 α/β ratio). Separation of the α- and β-anomers was achieved using GPC (Biogel P2 column).

2.4.2. Synthesis of β-(1,2)-mannan oligosaccharides with a modified reducing end

Enzymatic synthesis of β-(1,2)-mannooligosaccharides bearing methyl-3-(α-D-mannopyranosylthio)propanoate as the reducing end residue, was achieved following the previously described protocol (section 2.1) (Scheme 1) which has been optimized for the generation of mannooligosaccharides with DP up to 4. The modified protocol consisted in using 3 equivalents of Man-1-P as the donor and 1 equivalent of **4a** as the acceptor in the presence of 0.3 mg mL^{-1} of enzyme in sodium acetate buffer (200 mM, pH 5.0) at 37 °C for 30 min. The reaction was monitored by TLC analysis and terminated by heating at 95 °C for 15 min (Scheme 3) Analysis of the reaction mixture by MALDI-TOF MS revealed the presence of a series of signals with *m/z* values in the agreement with the calculated masses for sodium adducts of mannooligosaccharides with DP2-DP8.

The desired products were isolated by GPC using a Biogel P2 column to give the disaccharide **5** (25%), the trisaccharide **6** (14%) and the tetrasaccharide **7** (7.1%). By comparison with the previous synthetic approach, using a mannose acceptor, with a modified reducing end, resulted in lower DP level of the final products, (Figs. S8 and S9). Electrospray ionisation-mass spectrometry (ESI-MS) confirmed DP of purified mannooligosaccharides by the presence of $[M+Na]^+$ adduct at *m/z* 467.13 (disaccharide **5**), *m/z* 629.0 Da (trisaccharide **6**), and *m/z* (tetrasaccharide **7**).

NMR analysis and assignment for β-(1,2)-mannotrioside **6** and β-(1,2)-mannotetraoside **7** was supported by HSQC spectra as described for compounds **1** and **2**. Clear resolution of the HSQC spectra of the anomeric region of the β-(1,2)-mannotriose **6** allowed the assignment of the reducing end signals of $H_1^A\beta$ at 5.37, $H_1^C\alpha$ at 4.80 and $H_1^B\alpha$ at 4.79 ppm, respectively. These signals correlated with ^{13}C signals at 100.7, 98.0 and 82.8 ppm for C_1^B, C_1^C and C_1^A. The NMR spectra of β-(1,2)-mannotetraose **7** α-anomer identified by a ^{1}H NMR signals at 5.46, 4.95, 4.92 and 4.87 ppm, correlating with ^{13}C NMR signal at 101.1, 100.9, 98.2, 82.8 ppm for C_1^C, C_1^D, C_1^B and C_1^A, respectively.

3. Conjugation of methyl 3-[β-D-(1,2)-mannosyl-β-D-(1,2)-mannosyl-α-D-mannosylthio] propanoate (6) to BSA carrier protein

β-(1,2)-Linked mannotrioside **6** was selected as a suitable candidate for a case point conjugation reaction to BSA [20]. The terminal methyl ester in **6** was converted to an acyl hydrazide followed by *in situ* conversion into the reactive acyl azide to target the exposed lysine residues on the BSA Following a methodology established in the earlier work from Lemieux and co-workers [17], β-(1,2)-mannotrioside **6** was reacted with hydrazine monohydrate in ethanol at 55 °C for 4 h. The conversion of the methyl ester in to the acyl hydrazide [18] was confirmed by ^{1}H NMR analysis showing the disappearance of the methoxy singlet signal at 3.61 ppm. Using basic pH conditions it is an important factor for a successful conjugation which is dependent on the primary amines of lysine residues being in the free base form (Fig. 4) [19]. After

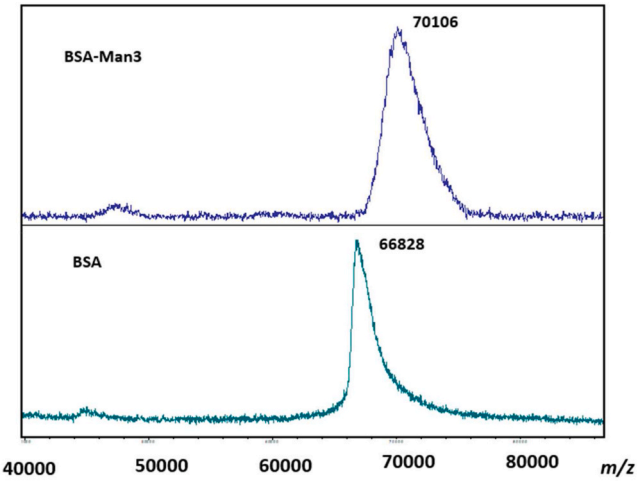
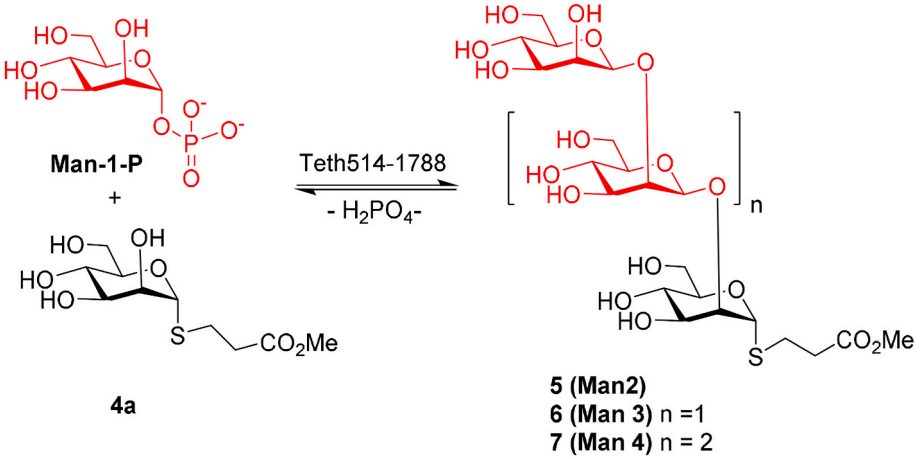


Fig. 4. MALDI-TOF mass spectrometry analysis of BSA pre- and post-conjugation with β-1,2-mannan oligosaccharide **6**.



Scheme 3. Enzymatic synthesis of β-(1,2)-mannans with modified reducing end catalysed by a β-(1,2)-oligomannan phosphorylase.

purification by dialysis the BSA conjugate was analysed by MALDI-TOF mass spectrometry (Fig. 4). The molecular weight difference between conjugated and unconjugated BSA accounts for 3278 Da (70106 Da–66828 = 3278 Da), each Man-3 contributes for 573 Da to the increase in molecular weight, which estimates an average of 5.7 residues per protein 3278 Da/573 Da = 5.7).

4. Conclusions

Herein we report a convenient β-1,2-oligomannan phosphorylase-mediated synthesis of β-(1,2)-oligomannoside of Man3 and Man4, capped at the reducing terminus with a functional tether suitable for protein conjugation, to support immunological evaluation of the resulting glycoconjugates. In contrast, efforts to synthesise reducing oligo-β-1,2-manann oligosaccharide and subsequently introduce an anomeric azide functionality were complicated by the formation of an inseparable anomeric mixtures. Fortunately, however, while the natural acceptor for the Teth514_1788 β-1,2-oligomannan phosphorylase is a β-1,2-linked mannose unit, the enzyme demonstrated to be tolerant towards other anomeric functionality and configuration, making it a flexible addition to the β-mannan synthesis armoury.

Following the successful enzymatic synthesis and GPC-based purification of β-1,2-linked mannotrioside **6** an illustrative neo-glycoconjugate was prepared yielding a BSA decorated with an average of 5.7 mannotrioside allowing a proof of concept workflow for vaccine candidates.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carres.2023.108807.

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