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Investigating the candidacy of lipopolysaccharide-based glycoconjugates as vaccines to combat *Mannheimia haemolytica*

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Abstract Inner core lipopolysaccharide (LPS) has been shown to be conserved in the majority of veterinary strains from the species *Mannheimia haemolytica*, *Actinobacillus pleuropneumoniae* and *Pasteurella multocida* and as such is being considered as a possible vaccine antigen. The proof-in-principle that a LPS-based antigen could be considered as a vaccine candidate has been demonstrated from studies with monoclonal antibodies raised to the inner core LPS of *Mannheimia haemolytica*, which were shown to be both bactericidal and protective in a mouse model of disease. In this study we confirm and extend the candidacy of the inner core LPS by demonstrating that it is possible to elicit functional antibodies against *Mannheimia haemolytica* wild-type strains following immunisation of rabbits with glycoconjugates elaborating the conserved inner core LPS antigen. The present study describes a conjugation strategy that uses amidases produced by *Dictyostelium discoideum*, targeting the amino functionality created by the amidase activity as the attachment point on the LPS molecule. To protect the amino functionality on the phosphoethanolamine (PEtn) residue of the inner core, we developed a novel blocking and unblocking strategy with t-butyl oxycarbonyl. A maleimide-thiol linker strategy with the thiol linker on the carboxyl residues of the carrier protein and the maleimide linker on the carbohydrate resulted in a high loading of carbohydrates per carrier protein. Immunisation derived antisera from rabbits recognised fully extended *Mannheimia haemolytica* LPS and whole cells from serotypes 1 and 2, despite a somewhat immunodominant response to the linkers also being observed. Moreover, bactericidal activity was demonstrated to a strain elaborating the immunising carbohydrate antigen and crucially to wild-type cells of serotypes 1 and 2, thus further supporting the consideration of inner core LPS as a potential vaccine antigen to combat disease caused by *Mannheimia haemolytica*.

Keywords *Mannheimia haemolytica* · Conjugate vaccine · LPS

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Introduction

Mannheimia haemolytica is a Gram-negative bacterium and is primarily a bovine and ovine pathogen [1]. It is considered as the most important bacterial pathogen of those that form the bovine respiratory disease (BRD) complex and causes major economic losses to the farming industry, notably beef and dairy cattle farmers [2]. *M. haemolytica* was originally classified as *Pasteurella haemolytica* and divided into two biotypes (A and T) and 16 serotypes [3], and subsequently the *P. haemolytica* complex was sub-divided and the species *M. haemolytica* was proposed [4]. The majority of diseases in cattle and sheep are caused by infections with serotypes 1 and 2 [5]. It has also been noted that diseases in sheep are from isolates that elaborate LPS with an O-antigen, whereas the majority of *M. haemolytica* isolates causing disease in cattle do not elaborate a LPS with an O-antigen [5]. There are several virulence factors produced by this organism that include outer membrane proteins (adhesins), capsular polysaccharides, secreted toxins (leukotoxin) and lipopolysaccharide

(LPS) [6–9]. The latter two factors have been postulated to complex and in such a way increase leukotoxin activity [10]. Antibodies to leukotoxin have been shown to protect cattle to respiratory challenge, although the antibodies are neutralizing the toxin and not eradicating the bacteria [11]. It has been postulated that the most effective vaccines to combat *M. haemolytica* disease would be a combination, promoting both neutralizing antibodies to the leukotoxin and antibodies to a cell surface target such as outer membrane proteins or lipopolysaccharides. A good cell surface vaccine antigen would be conserved, genetically stable, accessible to host immune responses and the immune response to the antigen would be protective. Studies on the outer membrane protein PlpE have shown great promise as a vaccine candidate, however OMPs are renowned for their ability to vary their surface exposed domains and thus evade the induced immunity from prior vaccination [12]. Our approach has been to examine the potential of the LPS molecule as a vaccine candidate. LPS consists of three regions, a lipid A region that links the LPS molecule to the bacterial surface via fatty acid residues, a relatively conserved core oligosaccharide region, which links the lipid A region to the third region, the variable polysaccharide antigen (O-antigen). Strain heterogeneity in capsular and O-antigenic polysaccharides would probably preclude these structures as economically viable vaccine candidates due to their ability to provide coverage only to the homologous strain or to strains producing an identical structure. Alternatively, if a conserved core LPS structure could be identified, this may have a utility as a vaccine candidate that would provide broad coverage to all strains. Previous and ongoing structural analyses of the LPS structures of three important veterinary species, *M. haemolytica*, *Pasteurella multocida* and *Actinobacillus pleuropneumoniae* in our laboratories had identified a conserved inner core LPS structure in all strains so far investigated; *A. pleuropneumoniae* [13], *P. multocida* [14–16] and *M. haemolytica* [17]. We have recently shown that the bovine and ovine serotype 2 genome strains [18] also elaborate the conserved inner core LPS structure [19]. The proof-in-principle that a LPS-based antigen could be considered as a vaccine candidate to combat veterinary disease has been demonstrated from studies with monoclonal antibodies raised to the inner core LPS of a mutant strain of *M. haemolytica*, termed *losB*, elaborating the conserved inner core structure, which were shown to be both bactericidal and protective in a mouse model of disease [20]. We have shown previously in our studies with the human pathogen *Neisseria meningitidis* that it is possible to prepare glycoconjugates, where the LPS-derived carbohydrate vaccine antigen is linked to a carrier protein and can generate an immune response to the carbohydrate component. We showed that antibodies derived in this way were bactericidal and protective against wild-type *N. meningitidis* strains [21]. Similarly, we and others have derived polyclonal sera following immunisations in rabbits with glycoconjugates based on a conserved inner core LPS structure from *Moraxella catarrhalis* that were broadly cross-reactive and able to facilitate bactericidal killing [22, 23]. In this study we confirm and extend the candidacy of the inner core LPS by demonstrating that it is possible to elicit functional antibodies against *M. haemolytica* wild-type strains following immunisation of rabbits with glycoconjugates elaborating the conserved inner core LPS antigen found in several veterinary species.

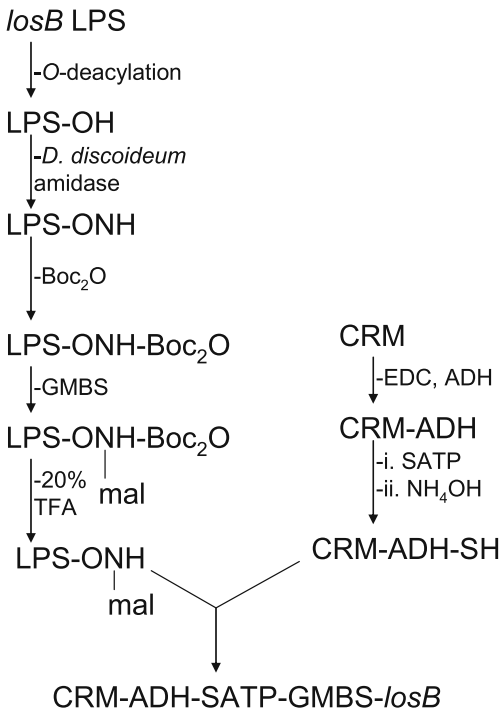
Materials and methods

Growth of bacteria and preparation of purified LPS

M. haemolytica strain *losB* (NRCC # 6306) where the *losB* gene has been disrupted by the insertion of a chloramphenicol resistance cassette, was grown and the LPS isolated as described previously [20].

Preparation of conjugates from purified LPS

The glycoconjugate was prepared as described below and as illustrated in the following scheme:-



O-deacylation

Purified LPS was treated with anhydrous hydrazine as described previously to prepare *O*-deacylated LPS (LPS-

OH) [21]. The composition of the LPS-OH was confirmed by CE-ES-MS.

N-deacylation

LPS-OH was the substrate for amidase enzymes produced by *Dictyostelium discoideum* under starvation conditions in order to prepare *N*-deacylated, *O*-deacylated LPS (LPS-ONH). *D. discoideum* cells of strain AX3 were grown axenically in liquid nutrient medium [24] at 22°C to a density of $2–3\times10^6$ cells/ml. The cells were pelleted, resuspended and washed twice in Sorensen's buffer (14.6 mM KH_2PO_4, 2 mM Na_2HPO4, pH 6.3). The cells were resuspended at 2×10^8 cells/3 ml of Sorensen's buffer and transferred to a conical flask. 2 mg of LPS-OH were added for each 3 ml of 2×10^8 *D. discoideum* cells and incubated at 24°C overnight with shaking (120 rpm). The cells were pelleted (3 K, 2 min.) and the resulting supernatant was pelleted (13 K, 30 min.). This supernatant was passed through a 10 KDa molecular mass cut-off filter, MMCF (Amicon ultra-15, Millipore), at 4000 × g at 4°C for 20 min. The MMCF was washed twice with 15 ml of distilled water (centrifuge as above). The flow through sample which contains *N*-deacylated LPS-OH was lyophilised. The lyophilised material was eluted from a Sephadex G-25 (Amersham 17-0033-02, medium) column and lyophilised.

Protection of PEtn residue

The LPS-ONH (4 mg/ml) was dissolved in H_2O with 8 molar equivalents of NaOH and 0.6 molar equivalents of di-*tert*-butyl dicarbonate (Boc_2O) (Aldrich) was added dissolved in the same volume of DMSO (BDH Chemicals) and stirred for 75 min. at 22–24°C. The mixture was extracted 3X with ethyl acetate (6 ml/10 mg CHO). The aqueous layer was lyophilised. The sample was eluted from a Sephadex G-25 column, eluting with water and the product peak was lyophilised. The product was monitored by CE-ES-MS.

Attachment of linker molecule

The Boc_2O -protected carbohydrate (4 mg/ml) was dissolved in 10X Dulbecco's PBS (Gibco) and a 1x molar equivalent of sulfo-GMBS (*N*-[g-Maleimidobutyryloxy] sulfosuccinimide ester, Pierce) was added every 20 min. until 5 molar equivalents were added. The pH was adjusted to 7.4 and monitored every 10 min adjusting as required with 0.1 M NaOH and the reaction was allowed to proceed for 2 h. The sample was eluted from a Sephadex G-25 column, eluting with water and the product peak was lyophilised. The product was monitored by CE-ES-MS as detailed below.

De-protection of PEtn residue

The Boc_2O -protected carbohydrate molecule with the incorporated linker was dissolved in 20% trifluoroacetic acid (TFA, Fisher Scientific) at 6 mg/ml and left at 22–24°C for 2 h. The sample was eluted from a Sephadex G-25 column, eluting with water and the product peak was lyophilised. The product was monitored by CE-ES-MS as detailed below.

Activation of protein carrier and conjugation

In order to conjugate the protein carrier molecule CRM_{197} to the maleimide-tagged carbohydrate it was necessary to modify the carboxyl groups on the CRM_{197} protein (5 mg in 2 ml of 100 mM 2-(*N*-morpholino) ethanesulfonic acid (MES, Aldrich) at pH 5.2) by reaction with an 1800 × molar excess of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Pierce) and a 1500 × molar excess of adipic acid dihydrazide, (ADH, Pierce) in 2 ml of 100 mM MES, pH 5.2 for 18 h at 4°C. The product was purified on an Ultra-15 10 kDa cut off spin column (Millipore), washing with 100 mM sodium phosphate pH 6.8×3, and concentrated to approximately 0.5 ml and stored at 4°C. The activated protein was characterised by MALDI-MS as described below. The ADH activated CRM_{197} was diluted to 2 ml in 100 mM sodium phosphate, adjusting the pH to 6.0, and reacted with a 100 × molar excess of *N*-succinimidyl-*S*-acetylthiopropionate (SATP, Pierce) dissolved in 0.1 ml of DMSO. This was reacted for 45 min at 22–24°C in the dark. The sample was purified on a G-25 column, eluting with 100 mM sodium phosphate pH 6.8. The product peak was concentrated to approximately 0.5 ml using a 10 KDa MMCF (Amicon ultra-15, Millipore) and stored at 4°C. The activated protein was characterised by MALDI-MS as described below.

The acetate group protecting the thiol moiety of the SATP residue was removed in a 1.5 mg/ml 100 mM sodium phosphate pH 6.8 solution containing 100 mM hydroxylamine hydrochloride (JT Baker) at 22–24°C for 1.5 h. The sample was purified on a G-25 column, eluting with 100 mM sodium phosphate pH 6.8. Protein positive fractions were pooled and concentrated using a 10 KDa MMCF (Amicon ultra-15, Millipore) and stored at 4°C.

For the conjugation reaction the maleimide activated carbohydrate (25X molar excess) and thiol activated protein (0.5 mg/ml) were left to react at 22–24°C for 1 h at pH 6.8 in 100 mM sodium phosphate, in the dark, under N_2, with gentle shaking (50 rpm). A second addition of carbohydrate was then provided so that a 50 x molar excess of carbohydrate was used in total. The reaction was allowed to continue for 3 h at 22–24°C as above and then stored for

18 h without shaking at 4°C. The conjugation reaction mixture was concentrated to~1 ml. as described above in order to remove free carbohydrate. The concentrate was washed and concentrated a further four times using Dulbecco's PBS (Gibco) containing 10 mM sodium citrate (Sigma). The final concentrate was stored at 4°C. The glycoconjugate was characterised by MALDI-MS, SDS-PAGE and Western blotting as described below.

Analytical methods

Sugars were determined as their alditol acetate derivatives by GLC-MS as described previously [25].

Mass spectrometry and nuclear magnetic resonance spectroscopy

Capillary electrophoresis electrospray mass spectrometry (CE-ES-MS), and NMR spectroscopy were performed as described previously [26]. Matrix-assisted laser desorption ionization- time of flight (MALDI-TOF) mass spectra were obtained using a Voyager DE-STR mass spectrometer (Applied BioSystems, Foster City, CA, U.S.A.). The instrument was operated in positive, linear ion mode under delayed extraction conditions (200 ns) using an accelerating voltage of 25 000 V. Each spectrum is the average of approximately 100 laser shots. The matrix used was 3,5-dimethoxy-4-hydroxy cinnamic acid (sinapinic acid), prepared at a concentration of 10 μg/μl in 30% acetonitrile and 0.1% formic acid (v/v). These solutions were spotted directly on the MALDI target in a 1:3 ratio with matrix.

SDS-PAGE and Western blotting

The conjugates were separated on 10% Tris–HCl pre-cast gels under reducing conditions with the buffer system of Laemmli [27]. SDS-PAGE was stained with Bio-Safe Coomassie, and an unstained duplicate was trans-blotted to polyvinylidene fluoride (PVDF) membranes. Membranes were immuno-stained with carbohydrate specific Abs, followed by alkaline phosphatase conjugated anti-rabbit IgG and developed with Alkaline Phosphatase Substrate Kit (Bio-Rad).

Immunisation

Three New Zealand white rabbits were immunised subcutaneously with the glycoconjugate. Each rabbit received 50 μg of conjugated carbohydrate as 2×0.5 ml per immunisation with Freunds adjuvant for the prime injection and incomplete Freunds adjuvant for the boosts. The rabbits were boosted on day 28 and 56 and sera recovered following trial bleed on day 42 and terminal heart puncture on day 70. Two rabbits also received control immunisations, which consisted of the *D. discoideum* deacylated carbohydrate (50 μg per rabbit) admixed with the same amount of protein (CRM_{197}) as in the glycoconjugate and appropriate adjuvant.

LPS ELISA

Purified and well-characterized wt and mutant LPS were used in solid-phase indirect ELISA to determine the binding profiles displayed by the rabbit sera as described previously [21].

Whole cell ELISA

Wells of Nunc Maxisorp EIA plates were coated with 100 μl of formalin-killed bacteria (optical density at 620 nm [OD620] of 0.080) in H_2O for 18 h in a 37°C drying oven and then brought to 22–24°C before use. Plates were blocked with 1% bovine serum albumin (BSA)-PBS for 1 h at 22–24°C, wells were washed with PBS–0.05% Tween 20 (PBS-T), and incubated with rabbit sera for 3 h at 22–24°C. Following washing with PBS-T, alkaline phosphatase-labeled goat anti-mouse IgG (Cedarlane Laboratories) diluted 1:3,000 in 1% BSA-PBS was added for 1 h at 22–24°C. The plates were then washed and developed with Phosphatase Substrate System (Kirkegaard and Perry Laboratories). After 60 min OD was measured at *A*405 nm using a microtiter plate reader.

Bactericidal assay

Pre-immune and day 70 sera from rabbits RVMh2 and RVMh3 were used as the source of sera for the BCA. Ascites fluid from mAb MhG8 [15] was used as a positive control (data not shown). The assay was performed according to the method of Sutherland [28]. Briefly, *M. haemolytica* cells were grown to a growth level of 5 x10^3 cfu/ml in 10 ml BHI broth. Cells were pelleted at 5000 × g, and washed twice in D-PBS (containing Ca and Mg [Gibco # 14040–133]). The final cell pellet was resuspended in 10 ml D-PBS. Test sera were heat-inactivated at 56°C for 30 min. to destroy endogenous complement. Assays were set-up in triplicate in 96-well, tissue culture grade, flat-bottomed micro-titre plates (NUNC). 20 μl of the appropriate dilution of antisera in D-PBS was added to each well followed by the bacterial suspension (100 μl) and the plate incubated for 15 min. at RT. Complement (80 μl, baby rabbit, #CL3441-S, Cedarlane) was added to each well, and incubated covered at 37°C for 30 min. The reaction was terminated by placing the plate on ice, and triplicate samples (50 μl) from each well were plated

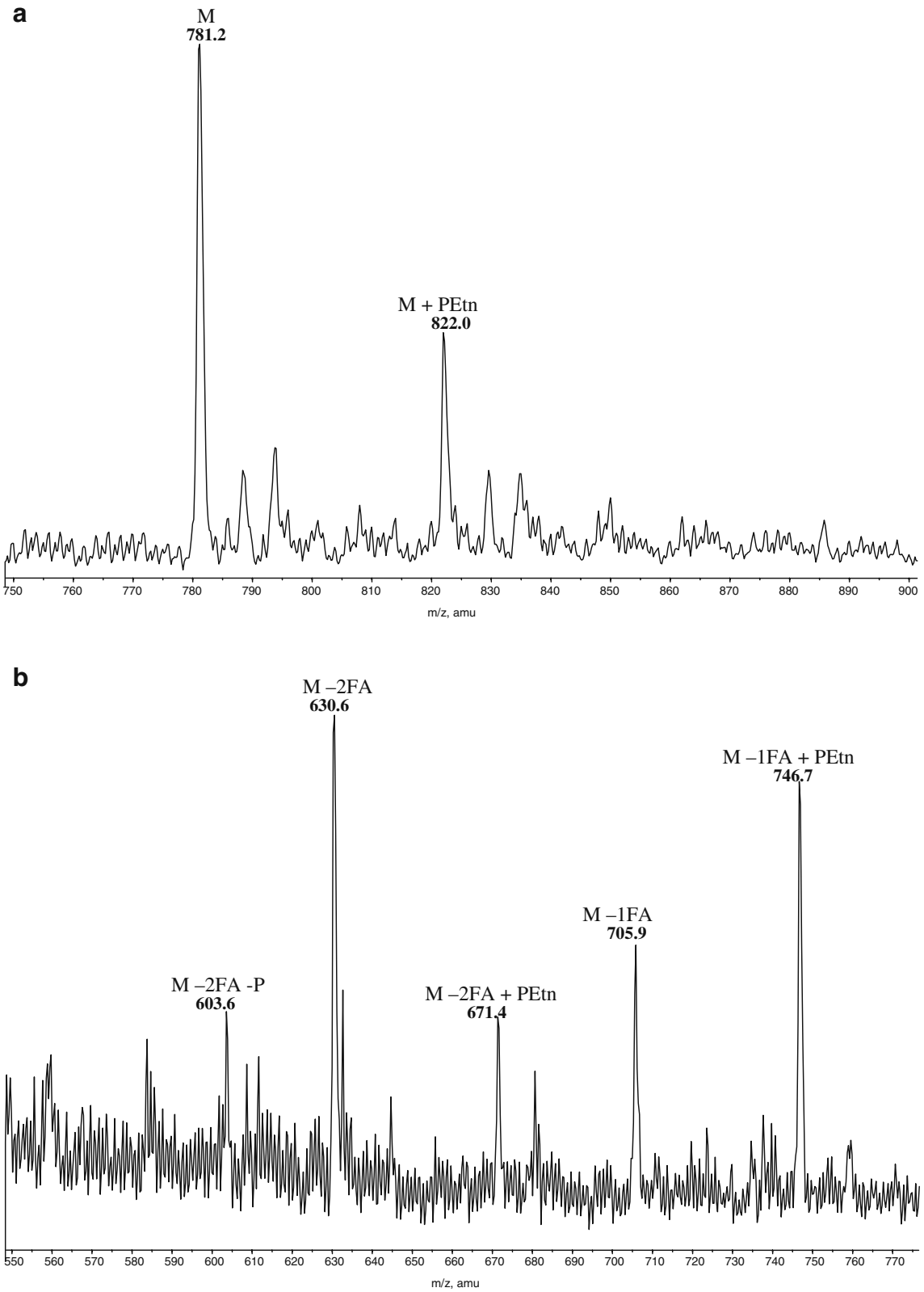


Fig. 1 CE-ES-MS analyses of *O*-deacylated LPS from *Mannheimia haemolytica* **a** before; **b** after treatment with amidase from *Dictyostelium discoideum*; **c** after *t*-Boc protection of PEtn residue of the *losB* derived material. M is the *O*-deacylated LPS molecule with a composition of 4Hep, 2Hex, Kdo-P, Lipid A-OH. Presence or absence of phosphoethanolamine (PEtn), fatty acid residues (FA), and *t*-Boc protecting group (Boc) residues are indicated

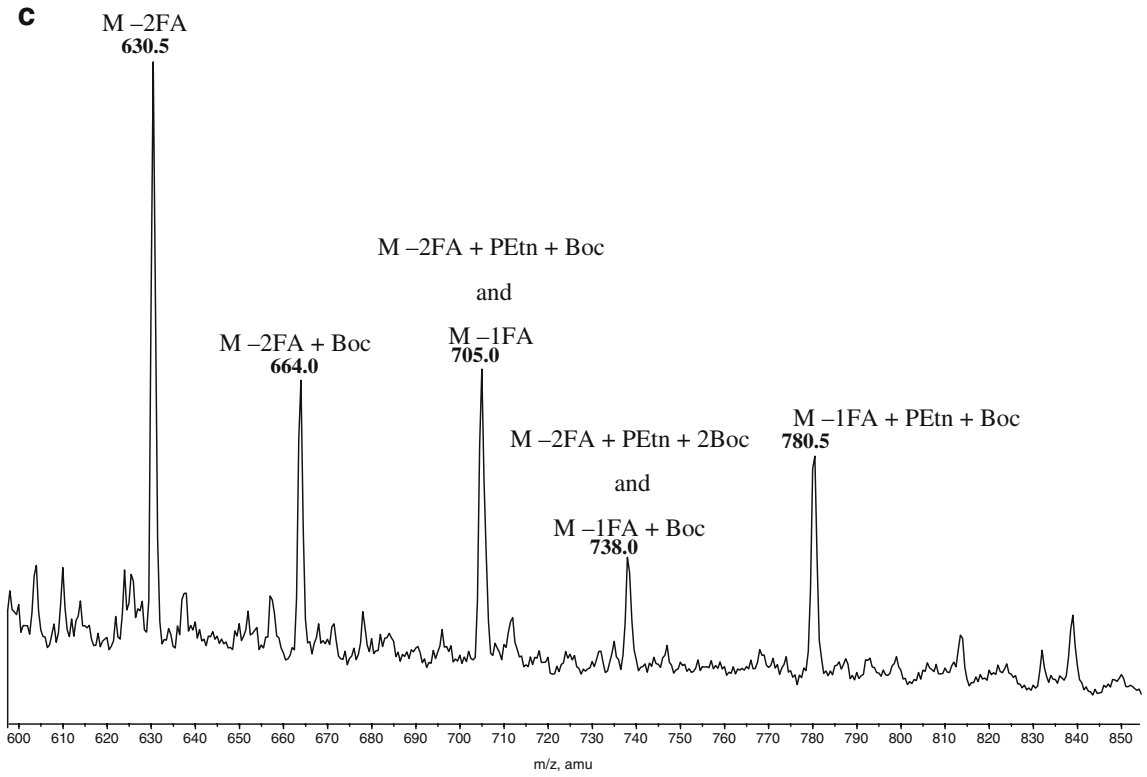


Fig. 1 (continued)

on BHI agar plates and incubated at 37°C for 18 h. Control wells containing bacterial suspension alone, bacterial suspension with complement, and bacterial suspension with appropriate dilution of antibody were also included in each assay. The bacterial growth was counted and the percentage growth relative to the bacterial suspension with complement was calculated.

Results

Carboxyl targeted conjugates were prepared as detailed in the Materials and Methods and each step of the strategy was quality controlled by MS and or NMR as appropriate.

Characterisation of *D. discoideum* amidase treated LPS-OH

The extent and specificity of *N*-deacylation achieved during treatment with *D. discoideum* amidases was effectively monitored by MS (Fig. 1). LPS-OH of *M. haemolytica losB* gave a spectrum (Fig. 1a) containing two major triply charged ions of *m/z* 781.2^{3-} and 822.0^{3-} corresponding to a composition of 4Hep, 2Hex, Kdo-P, Lipid A-OH with variable substitution with a PEtn residue at the Kdo-P. As can be seen in Fig. 1b treatment of *M. haemolytica losB* LPS-OH with *D. discoideum* amidase results in the loss of two or one N-linked fatty acids as illustrated by the triply charged ions of *m/z* 630.6^{3-} and 705.9^{3-}. Correspondingly each of these glycoforms is variably substituted with a PEtn residue as evidenced by triply charged ions at *m/z* 671.4^{3-} and 746.7^{3-}. The ion at *m/z* 603.6^{3-} corresponds to the loss of a phosphate residue from the completely deacylated molecule.

Protection of PEtn residue

Following *N*-deacylation, the PEtn residue on the 3-deoxy-D-*manno*-oct-2-ulosonic acid-phosphate (Kdo-P) moiety of the *losB* derived molecule was protected with Boc_2O, and the product was characterised by MS (Fig. 1c). MS analysis of the material before (Fig. 1b) and after (Fig. 1c) Boc_2O protection illustrated that one Boc_2O moiety had been incorporated onto the ions corresponding to the molecules elaborating a PEtn residue, by an increase in mass of 100 amu as evidenced by triply charged ions at *m/z* 780.5^{3-} and 705.0^{3-}, resulting from the protection of PEtn containing glycoforms at *m/z* 746.7^{3-} and 671.4^{3-} in the amidase treated material, respectively. Furthermore, some amino functionalities of the lipid A region had also been protected as evidenced by the triply charged ions at 738.0^{3-} and 664.0^{3-}, resulting from incorporation of a Boc_2O molecule on the triply charged ions of *m/z* 705.4^{3-} and 630.6^{3-} of the amidase treated material. Although this latter protection was not intended, the majority of amino

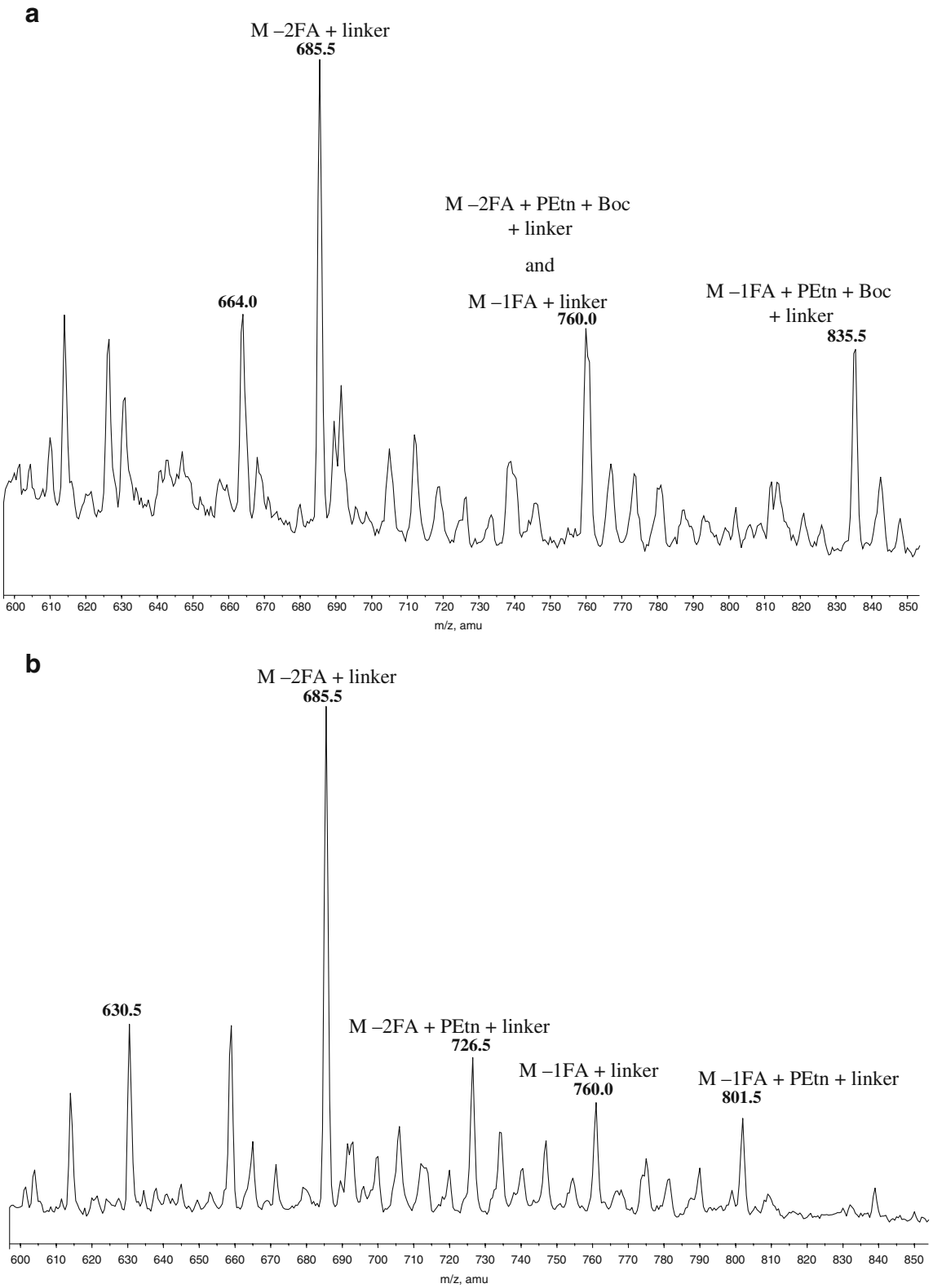


Fig. 2 CE-ES-MS analysis of linker incorporation and Boc protecting group removal; **a** introduction of a maleimide group to the *losB* Boc-protected molecule, **b** removal of Boc protecting group from the maleimide activated *losB* molecule. Presence or absence of phosphoethanolamine (PEtn), fatty acid residues (FA), *t*-Boc protecting group (Boc) and maleimide linker (linker) residues are indicated

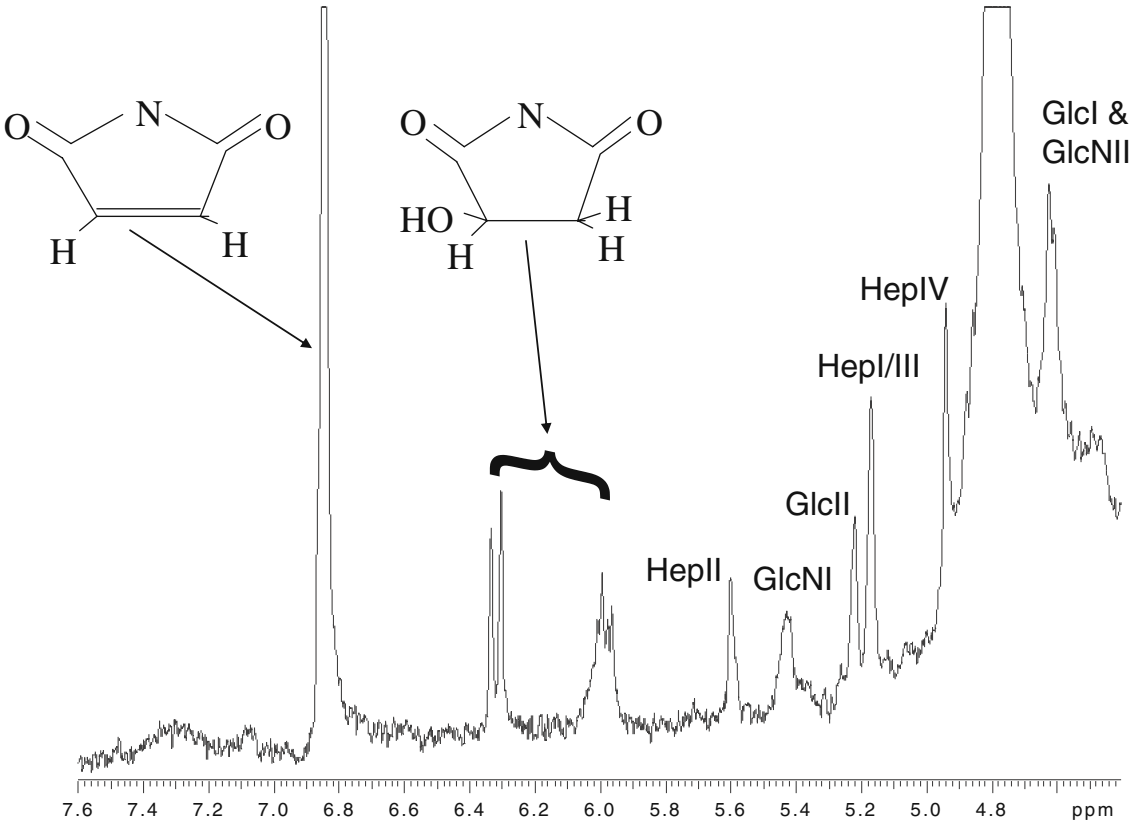


Fig. 3 Region of ^{1}H-NMR spectrum of maleimide activated *losB* molecule. Anomeric and linker related protons are detailed. Spectrum was recorded in D_2O at 25°C and referenced to the HOD signal at 4.78 ppm

functionalities in the lipid A region, the target for linker incorporation, had not been modified, as evidenced by the maintenance of ions corresponding to molecules without a PEtn residue (*e.g. m/z* 630.5^{3-}) compared to the disappearance of ions corresponding to molecules with a PEtn residue (*e.g. m/z* 746.7^{3-}) and by MS/MS studies (data not shown).

Attachment of linker

A maleimide containing linker was attached to the *losB* molecule as detailed in the material and methods and characterised by MS (Fig. 2a). An increase in mass of 165 amu was observed, as evidenced by the identification of triply charged ions (*m/z* 685.5^{3-}, 760.0^{3-}, 835.5^{3-}) consistent with the incorporation of the maleimide linker, when compared to the molecule without linker incorporation (Fig. 1c).

De-protection of PEtn residue

Following linker incorporation, the *t*-Boc blocking group was efficiently removed from the *losB* derivative by treatment with 20% TFA with no effect on the remainder of the molecule as characterised by MS (Fig. 2b). A decrease in mass of 100 amu was observed, as evidenced by the identification of triply charged ions (*m/z* 685.5^{3-}, 726.5^{3-}, 801.5^{3-}), consistent with the removal of the Boc protecting group, when compared to the Boc-protected molecule (Fig. 2a). Finally, the activated carbohydrate was examined by ^{1}H-NMR, which indicated that the maleimide linker was present on the carbohydrate molecule, by virtue of a sharp singlet at 6.8 ppm corresponding to the equivalent protons of the double bond of the maleimide ring (Fig. 3). Additionally anomeric proteins of the *M. haemolytica losB* inner core LPS were assigned along with evidence of a small amount of hydrolysis of the maleimide ring double bond.

Activation of protein carrier

Carboxyl groups of the chosen carrier protein, CRM_{197} were activated with a thiol containing linker (ADH-SATP) as described in the material and methods and characterised by MALDI-MS, which revealed that ~27 carboxyl residues had been activated with ADH as evidenced by a mass

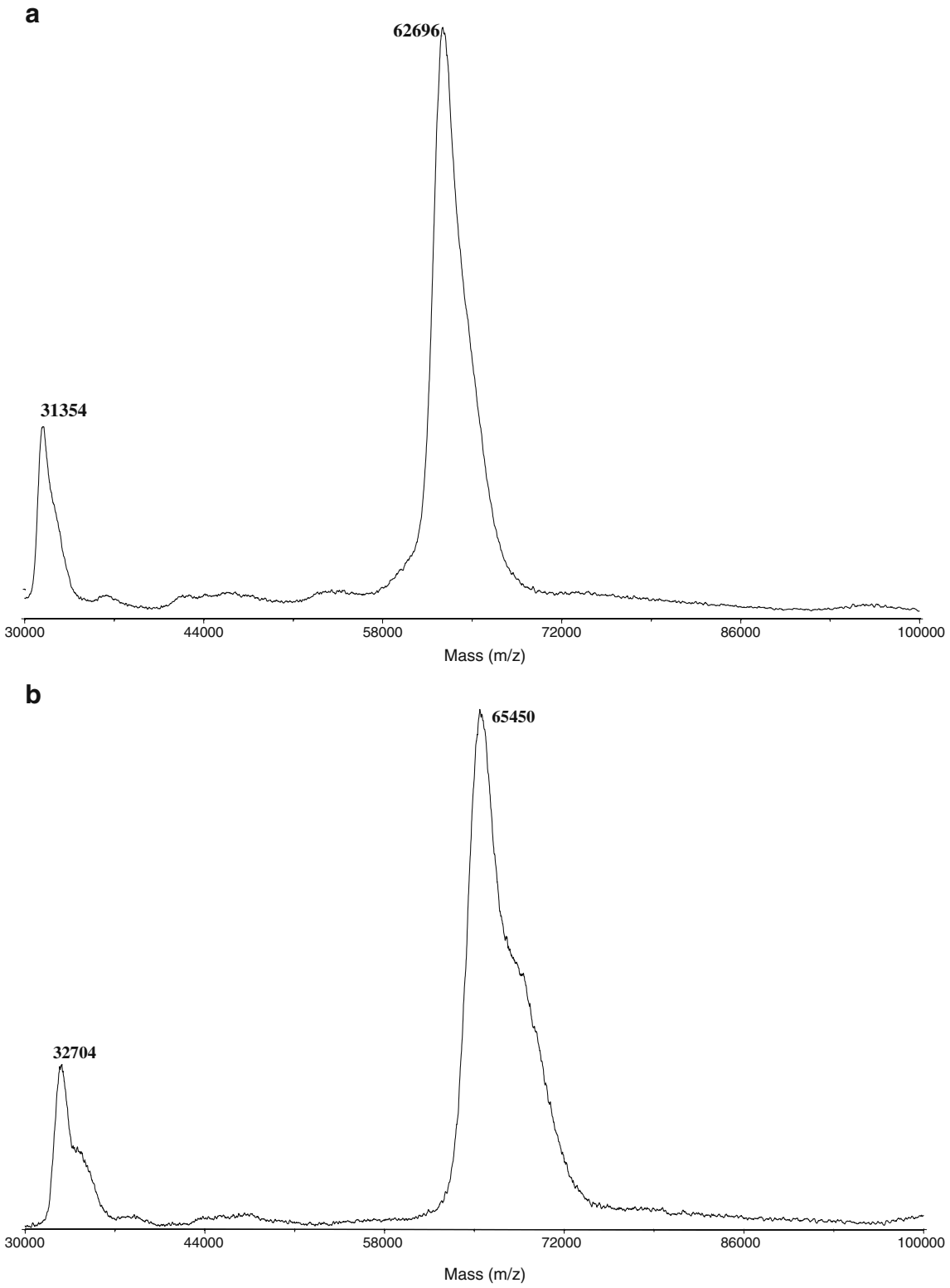


Fig. 4 MALDI-MS analyses of; **a** CRM, **b** CRM-ADH **c** CRM-ADH-SATP, **d** CRM-ADH-SATP-GMBS-*losB* conjugate

increase of ~4.2 kDa (Fig. 4a) over inactivated CRM_{197}. The ADH-activated CRM was then reacted with SATP in order to introduce a protected thiol moiety to the carrier protein and MALDI analysis determined that ~21 SATP residues had been added to the ADH groups as evidenced by a mass increase of ~2.8 kDa (Fig. 4b).

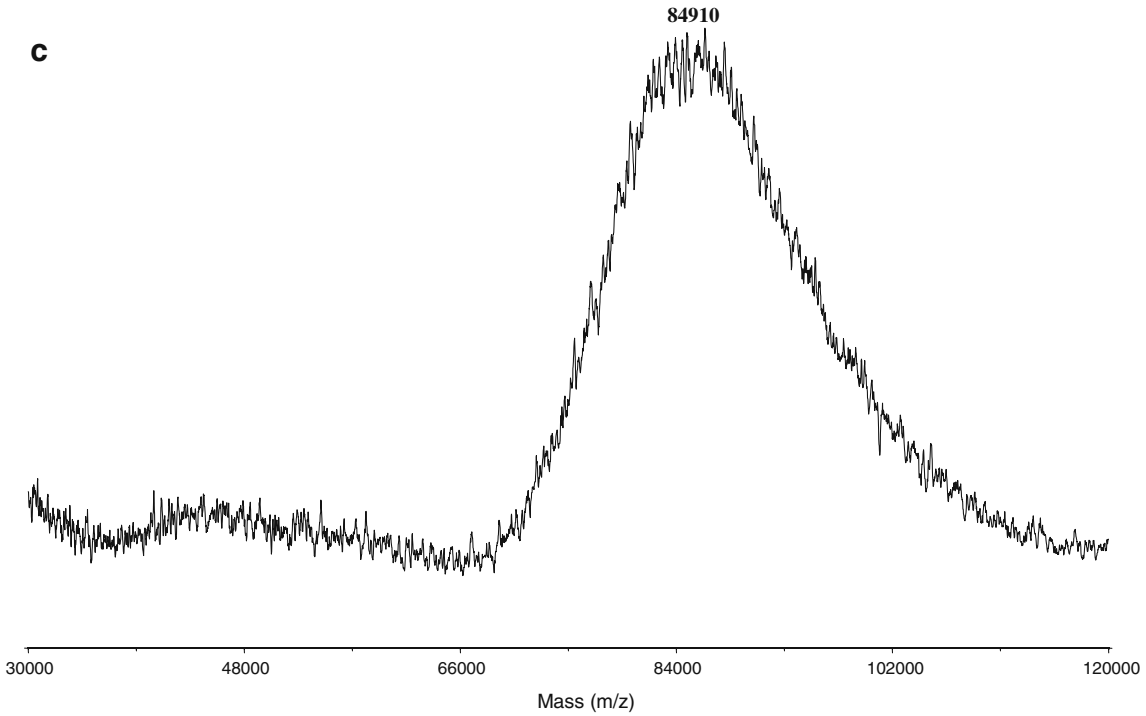


Fig. 4 (continued)

Characterisation of conjugation products

Following de-protection to expose the thiol moiety, activated CRM_{197} was conjugated to the carbohydrate as described above. Conjugation products were purified as described and monitored by MALDI-MS suggesting that~10 carbohydrate molecules had been attached per carrier protein (Fig. 4c), assuming a mass increase of~2,100 Da for each carbohydrate unit attached. SDS-PAGE and Western blotting revealed considerably slower migration of the conjugate than the activated protein carrier and recognition with a carbohydrate specific antibody (Fig. 5).

Immunogenicity of glycoconjugates

Rabbits were immunised, based on conjugated carbohydrate amounts, with a prime (complete Freunds adjuvant) and two boost (incomplete Freunds adjuvant) strategy of 50 μg of the CRM-ADH-SATP-*losB* conjugate. Rabbit sera were initially titrated against the homologous antigen, *M. haemolytica losB* LPS, which revealed good titers for sera from rabbits 2 and 3 (data not shown). These sera were subsequently shown to be broadly cross reactive against LPS from serotypes 1 and 2 of *M. haemolytica* (Fig. 6). There was also a significant and somewhat

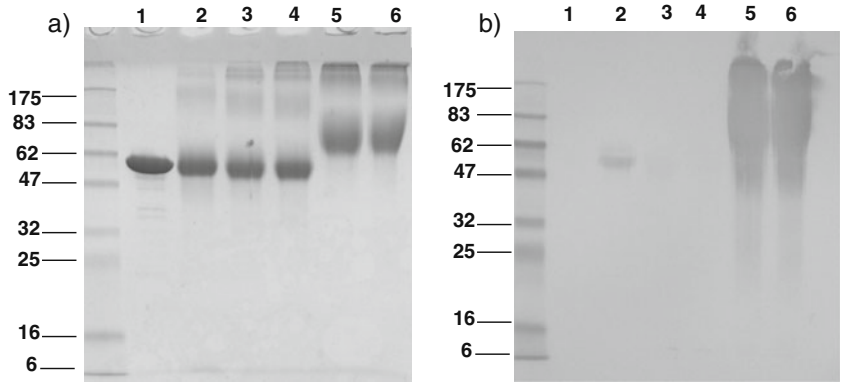


Fig. 5 Coomassie staining and Western blot analysis of CRM-ADH-SATP-GMBS-*losB* conjugate **a** Coomassie staining of samples after equal protein loading. **b** Western blot analysis of samples using RMC5 polyclonal antibody at a dilution of 1:5000. Lanes 1, 2, 3, 4, 5, 6 are CRM, CRM-ADH, CRM-ADH-SATP, CRM-ADH-SH, CRM-ADH-SATP-GMBS-*losB* conjugate after 1st CHO addition, CRM-ADH-SATP-GMBS-*losB* final conjugate respectively

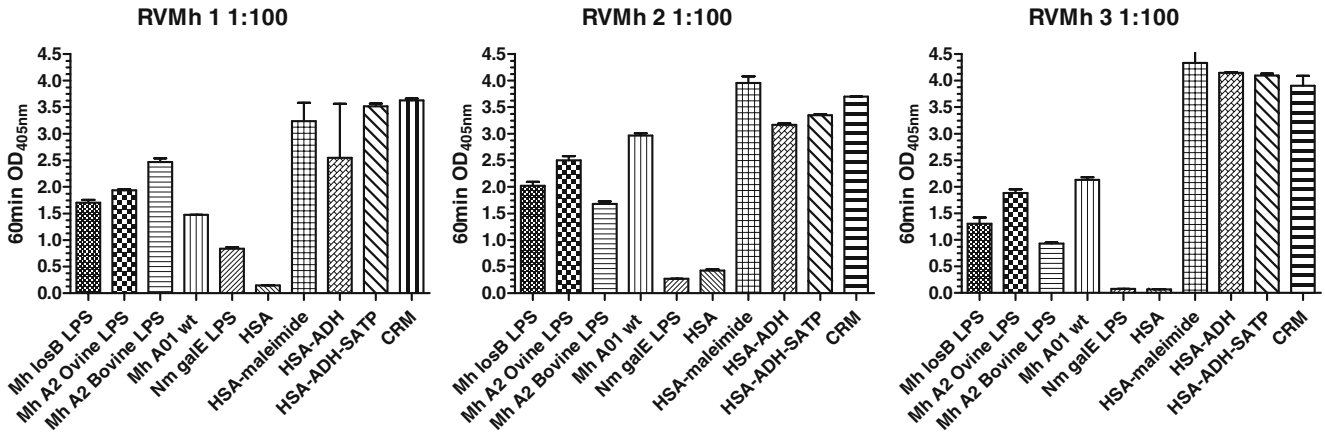


Fig. 6 ELISA cross-reactivity analysis of rabbit immune sera RVMh 1–3 against purified LPS from the indicated strains of *M. haemolytica* (Mh) and *Neisseria meningitidis* (Nm), carrier protein CRM, and also against the irrelevant protein, human serum albumin (HSA) with the linkers utilised in the conjugation strategy attached as indicated

immunodominant response to the linkers as revealed by testing against an irrelevant protein, human serum albumin (HSA) alone, or with the different linkers utilised in the conjugation strategy attached (Fig. 6). Final bleed sera from rabbits 2 and 3 recognised whole cells of both the homologous *M. haemolytica losB* and the wild-type *M. haemolytica* serotype 1 and 2 strains and the recognition from these rabbits was shown to be somewhat specific as control cells from the LPS-structurally non-related strain *Moraxella catarrhalis* were not recognised as well as the *M. haemolytica* cells (Fig. 7). Rabbit sera were then examined for their ability to facilitate complement mediated bactericidal killing of *M. haemolytica* cells. Post-immune sera were compared to pre-immune control sera from the same rabbits. Clear evidence of bactericidal killing was observed with sera from rabbits 2 and 3 against both the homologous *losB* mutant and the wt serotype 1 and 2 bovine strains (Table 1). We did not observe killing of the serotype 2 ovine strain, which was puzzling in the light of the comparable ELISA data of all the *M. haemolytica* strains, however we observed two colony morphologies for the ovine strain generated only on exposure to serum or complement, which complicated the interpretation of the bactericidal data for this strain.

Discussion

This study has described a conjugation strategy that has facilitated the preparation of a conjugate with a high loading of carbohydrate molecules per carrier protein. We originally developed this methodology to prepare conjugates with *N. meningitidis* inner core LPS [29] and subsequently with *M. catarrhalis* [22], and achieved a similar high loading illustrating the reproducibility of this strategy. The loading achieved can be qualitatively and quantitatively characterised by SDS-PAGE/Western and MALDI MS techniques, respectively. In employing the conjugation strategy described here, we did successfully utilise an amino functionality created at the reducing end of the carbohydrate by enzyme treatment with an amidase

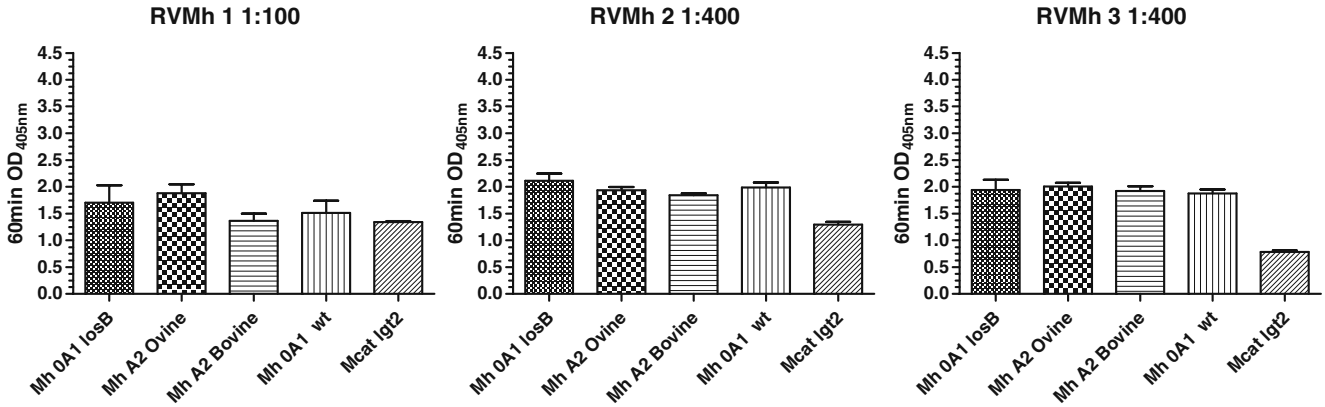


Fig. 7 ELISA analysis of rabbit immune sera RVMh 1–3 against whole cells from the indicated strains of *M. haemolytica* (Mh) and *Moraxella catarrhalis* (Mcat). Sera were diluted as indicated

Table 1 Bactericidal titers[1] of pre- and post-immunisation rabbit sera from *M. haemolytica losB* conjugate against *M. haemolytica* wild type and mutant strains. Baby rabbit complement was used as the source of complement at a dilution of 1/3.

Sera/Strain	Pre/Post	Serotype 1 *losB*	Serotype 1	Serotype 2 bovine	Serotype 2 ovine
losB-GMBS-SATP-ADH-CRM conjugate					
RVMh1	Pre	nk[2]	nk	nd[3]	nd
	Post D70	nk	nk	nd	nd
RVMh2	Pre	nk	nk	nk	nk
	Post D70	20	40	25	nk
RVMh3	Pre	nk	nk	nk	nk
	Post D70	80	40	25	nk

[1] Bactericidal titers expressed as the reciprocal of the serum dilution yielding>= 50% killing when compared to the corresponding pre-immune sera

[2] *nk* no killing

[3] *nd* not determined

from *D. discoideum*. This amino group could then be targeted as the site of conjugation whilst retaining the cyclic nature of the terminal glucosamine residue. However, this then presented the challenge of selectively protecting the amino functionality of the PEtn residue sometimes present on the Kdo-P in the inner core OS. We achieved this by utilising a simple blocking strategy, which preferentially attached to the inner core PEtn amino when ratios of blocking agent and substrate were carefully controlled. The lipid A amino functionalities were now available for reaction with the linker molecule, and the blocking group was then subsequently removed, thus directing the location of conjugation between the carbohydrate and carrier protein. Following immunisation, generally similar patterns of recognition were observed with each conjugate derived rabbit sera (RVMh 1–3) to both LPS and whole cells from the homologous *losB* antigen and the serotype 1 and 2 wild-type strains, however, the titers observed and specificity of the sera derived from rabbit 1 were lower as illustrated in the whole cell ELISA. We did observe a significant, somewhat immunodominant response to the maleimide and hydrazide containing linkers, and it appeared that at least 50% of the response was targeting the linkers as determined by ELISA studies with the irrelevant HSA protein elaborating the linkers. We believe that as long as titers to the target oligosaccharide are sufficient then an immune response to irrelevant epitopes would not be deleterious. This hypothesis is also supported by the fact that a commercial vaccine, the synthetic *Haemophilus influenz*ae capsular polysaccharide serotype B (Hib) vaccine, developed by Cuban scientists, contains the same maleimide linker as described here [30]. An immune response to the maleimide linker would be anticipated with the Cuban vaccine also, although this has not been reported, and clearly the Cuban vaccine has proven very effective in eradication of disease caused by Hib wherever it has been used. In this study although a large proportion of the immune response was apparently distracted to the maleimide linker, we still obtained antibodies capable of facilitating bactericidal killing, as serum bactericidal assays revealed that post-immune sera from two out of the three rabbits were capable of killing wt *M. haemolytica* cells at dilutions approaching 1:50 when compared to the pre-immune sera. The identification of two colony morphologies produced by only the ovine strain on exposure to serum or complement was perplexing and possibly affected the ability to obtain antibody-mediated bactericidal killing of this strain. We also observed a similar immune response to the linker molecules in our previous studies with LPS based vaccines for both *N. meningitidis* [31] and *M. catarrhalis* [22], and an ability to facilitate bactericidal killing of wild-type Moraxella strains, similar to that achieved in this study, was observed even in the presence of a response to the linkers, however, that was not the case with sera raised to meningococcal conjugates as only homologous strains were killed. Clearly the presence of an immune response to the linker molecules is not beneficial, but in the case of *M. haemolytica* and *M. catarrhalis* did not preclude facilitation of bactericidal killing. Nevertheless, this competition between hapten and linkers, especially for conjugates utilising smaller oligosaccharides as the carbohydrate component, is going to effect the maturation of the immune response to the desired carbohydrate target. A recent paper by Lipinski *et al.* [32] has introduced a novel alternative conjugation strategy, by utilising stereochemically variable linker molecules to suppress the immune response maturation to the linker moieties. The present study has therefore clearly confirmed and extended the potential of the inner core LPS as a vaccine candidate to combat disease caused by the significant veterinary pathogen *M. haemolytica*. Future studies are focusing

upon improvements to our conjugation strategies in order to reduce or avoid competing immune responses to linker molecules.

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References

1. Davies, R.L., Donachie, W.: Intra-specific diversity and host specificity within *Pasteurella haemolytica* based on variation of capsular polysaccharide, lipopolysaccharide and outer-membrane proteins. Microbiol. **142**, 1895–1907 (1996)
2. Whitely, L.O., Maheswaran, S.K., Weiss, D.J., Ames, T.R., Kannan, M.S.: *Pasteurella haemolytica* A1 and bovine respiratory disease: pathogenesis. J. Vet. Int. Med. **6**, 11–22 (1992)
3. Biberstein, E.L.: Biotyping and serotyping of *Pasteurella haemolytica*. Methods Microbiol. **10**, 252–269 (1978)
4. Angen, O., Mutters, R., Caugant, D.A., Olsen, J.E., Bisgaard, M.: Taxonomic relationships of the [*Pasteurella*] *haemolytica* complex as evaluated by DNA-DNA hybridizations and 16S rRNA sequencing with proposal of *Mannheimia haemolytica* gen. nov., comb. nov., *Mannheimia granulomatis* comb. nov., *Mannheimia glucosida* sp. nov., *Mannheimia ruminalis* sp. nov. and *Mannheimia varigena* sp. nov. Int. J. Syst. Bacteriol. **49**, 67–86 (1999)
5. Ali, Q., Davies, R.L., Parton, R., Coote, J.G., Gibbs, H.A.: Lipopolysaccharide heterogeneity in *Pasteurella haemolytica* isolates from cattle and sheep. J. Gen. Microbiol. **138**, 2185–2195 (1992)
6. Pandher, K., Confer, A.W., Murphy, G.L.: Genetic and immunologic analyses of PlpE, a lipoprotein important in complement-mediated killing of *Pasteurella haemolytica* serotype 1. Infect. Immun. **66**, 5613–5619 (1998)
7. McKerral, L.J., Lo, R.Y.: Construction and characterisation of an acapsular mutant of *Mannheimia haemolytica* A1. Infect. Immun. **70**, 2622–2629 (2002)
8. Shewen, P.E., Wilkie, B.N.: Cytotoxin of *Pasteurella haemolytica* acting on bovine leukocytes. Infect. Immun. **35**, 91–94 (1982)
9. Confer, A.W., Simons, K.R.: Effects of *Pasteurella haemolytica* lipopolysaccharide on selected functions of bovine leukocytes. Am. J. Vet. Res. **47**, 154–157 (1986)
10. Li, J., Clinkenbeard, K.D.: Lipopolysaccharide complexes with *Pasteurella haemolytica* leukotoxin. Infect. Immun. **67**, 2920–2927 (1999)
11. Shewen, P.E., Wilkie, B.N.: Vaccination of calves with leukotoxic culture supernatant from *Pasteurella haemolytica*. Can. J. Vet. Res. **52**, 30–36 (1988)
12. Ayalew, S., Blackwood, E.R., Confer, A.W.: Sequence diversity of the immunogenic outer membrane lipoprotein PlpE from *Mannheimia haemolytica* serotypes 1, 2 and 6. Vet. Microbiol. **114**, 260–268 (2006)
13. St. Michael, F., Brisson, J.-R., Larocque, S., Monteiro, M., Li, J., Jacques, M., Perry, M.B., Cox, A.D.: Structural analysis of the lipopolysaccharide derived core oligosaccharides of *Actinobacillus pleuropneumoniae* serotypes 1, 2, 5a and the genome strain 5b. Carbohydr. Res. **339**, 1973–1984 (2004)
14. St. Michael, F., Vinogradov, E.V., Li, J., Cox, A.D.: Structural analysis of the lipooligosaccharide from *Pasteurella multocida* genome strain Pm70 and identification of the putative lipopolysaccharide glycosyltransferases. Glycobiology **15**, 323–333 (2005)
15. St. Michael, F., Li, J., Vinogradov, E.V., Larocque, S., Harper, M., Cox, A.D.: Structural analysis of the lipopolysaccharide from *Pasteurella multocida* strain VP161: Identification of both Kdo-P and Kdo-Kdo species in the lipopolysaccharide. Carbohydr. Res. **340**, 59–68 (2005)
16. Stmichael, F., Harper, M., John, M., Stupak, J., Vinogradov, E., Parnas, H., Boyce, J.D., Adler, B., Cox, A.D.: Structural and genetic basis for the serological differentiation of *Pasteurella multocida* Heddleston serotypes 2 and 5. J. Bacteriol. **191**, 6950–6959 (2009)
17. Brisson, J.-R., Crawford, E., Uhrin, D., Khieu, N.H., Perry, M. B., Severn, W.B., Richards, J.C.: The core oligosaccharide component from *Mannheimia (Pasteurella) haemolytica* serotype Al lipopolysaccharide contains L-*glycero*-D-*manno* and D-*glycero*-D-*manno*-heptoses. Analysis of the structure and confirmation by high resolution NMR spectroscopy. Can. J. Chem. **80**, 949–963 (2002)
18. Lawrence, P.K., Kittichotirat, W., Bumgarner, R.E., McDermott, J. E., Herndon, D.R., Knowles, D.P., Srikumaran, S.: Genome sequences of *Mannheimia haemolytica* serotype A2: ovine and bovine isolates. J. Bacteriol. **192**, 1167–1168 (2010)
19. St. Michael, F., Vinogradov, E., Cox, A.D.: Structural analyses of the core oligosaccharide from the lipopolysaccharide of bovine and ovine strains of *Mannheimia haemolytica* serotype 2. Carbohydr. Res. **346**, 1333–1336 (2011)
20. Logan, S.M., Chen, W., Aubry, A., Gidney, M.A.J., Lacelle, S., St. Michael, F., Kuolee, R., Higgins, M., Neufeld, S., Cox, A.D.: Production of a D-*glycero*-D-*manno*-heptosyltransferase mutant of *Mannheimia haemolytica* displaying a veterinary pathogen specific conserved LPS structure and development and cross-reactivity of antibodies to this LPS structure. Vet. Microbiol. **116**, 175–186 (2006)
21. Cox, A.D., Zou, W., Gidney, M.A.J., Lacelle, S., Plested, J.S., Makepeace, K., Wright, J.C., Coull, P., Moxon, E.R., Richards, J. C.: Candidacy of LPS-based glycoconjugates to prevent invasive meningococcal disease: developmental chemistry and investigation of immunological responses following immunization of mice and rabbits. Vaccine **23**, 5045–5054 (2005)
22. Cox, A.D., St. Michael, F., Cairns, C.M., Lacelle, S., Filion, A., Neelamegan, D., Wenzel, C.Q., Richards, J.C.: Investigating the potential of conserved inner core oligosaccharide regions of *Moraxella catarrhalis* lipopolysaccharide as vaccine antigens: accessibility and functional activity of monoclonal antibodies and glycoconjugate derived sera. Glycoconjugate J. **28**, 165–182 (2011)
23. Ren, D., Yu, S., Gao, S., Peng, D., Petralia, R.S., Muszynski, A., Carlson, R.W., Robbins, J.B., Tsai, C.-M., Lim, D.J., Gu, X.-X.: Mutant lipooligosaccharide-based glycoconjugate vaccine demonstrates a broad-spectrum effectiveness against *Moraxella catarrhalis*. Vaccine **29**, 4210–4217 (2011)
24. Claviez, M., Pagh, K., Maruta, H., Baltes, W., Fisher, P., Gerisch, G.: Electron microscopic mapping of monoclonal antibodies on the tail region of *Dictyostelium* myosin. EMBO J. **1**, 1017–1022 (1982)
25. Sawardeker, D.G., Sloneker, J.H., Jeanes, A.: Quantitative determination of monosaccharides as their alditol acetates by gas liquid chromatography. Anal. Chem. **37**, 1602–1604 (1965)
26. Cox, A.D., Li, J., Brisson, J.-R., Moxon, E.R., Richards, J.C.: Structural analysis of the lipopolysaccharide from *Neisseria meningitidis* strain BZ157 *galE*: localisation of two phosphoethanolamine residues in the inner core oligosaccharide. Carbohydr. Res. **337**, 1435–1444 (2002)

27. Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**, 680–685 (1970)
28. Sutherland, A.D.: A rapid micro-method for the study of antibody-mediated killing of bacteria, with specific application to infection of sheep with *Pasteurella haemolytica*. Vet. Microbiol. **16**, 263–271 (1988)
29. Cox, A.D., St. Michael, F., Neelamegan, D., Lacelle, S., Cairns, C. M., Richards, J.C.: Investigating the candidacy of LPS-based glycoconjugates to prevent invasive meningococcal disease: chemical strategies to prepare glycoconjugates with good carbohydrate loading. Glycoconjugate J. **27**, 401–17 (2010)
30. Verez-Bencomo, V., Fernández-Santana, V., Hardy, E., Toledo, M. E., Rodríguez, M.C., Heynngnezz, L., *et al*.: A synthetic conjugate polysaccharide vaccine against *Haemophilus influenzae* type B. Science **305**, 522–525 (2004)
31. Cox, A.D., St. Michael, F., Neelamegan, D., Lacelle, S., Cairns, C. M., Giuliani, M.M., *et al*.: Investigating the candidacy of LPS-based glycoconjugates to prevent invasive meningococcal disease: immunology of glycoconjugates with high carbohydrate loading. Glycoconjugate J. **27**, 643–648 (2010)
32. Lipinski, T., Luu, T., Kitov, P.I., Szpacenko, A., Bundle, D.R.: A structurally diversified linker enhances the immune response to a small carbohydrate hapten. Glycoconjugate J. **28**, 149–164 (2011)