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PAPER

Synthesis and immunochemical evaluation of a non-methylated disaccharide analogue of the anthrax tetrasaccharide†

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Anthrax tetrasaccharide is an oligosaccharide expressed at the outermost surface of the *Bacillus anthracis* spores, featuring three rhamnoses and a rare sugar called anthrose. This motif has now been identified as a plausible component of future human vaccines against anthrax. We report herein the synthesis of a 2-*O*-demethylated-β-D-anthropyranosyl-(1→3)-α-L-rhamnopyranose disaccharide analogue of this tetrasaccharide from a cyclic sulfate intermediate. This disaccharide conjugated to BSA induces an anti-native tetrasaccharide IgG antibody response when administered in BALB/c mice. Moreover, induced sera bound to native *B. anthracis* endospores. These results suggest that the disaccharide analogue, easily amenable for a synthetic scale-up, could be used in a glycoconjugate antigen formulation.

Introduction

Bacillus anthracis, a Gram-positive, spore-forming soil bacterium, is the etiologic agent of anthrax. Anthrax is primarily a zoonotic disease but because of the ease of production, storage, and dissemination of the spores as well as the high lethality that results from spore exposure (mainly by inhalation), this pathogen has emerged as a potential biological warfare or bioterrorism agent. *B. anthracis* strains are sensitive to antibiotics. However, once commenced the treatment must be continued for prolonged periods and is usually ineffective for inhaled anthrax which is rarely recognized prior to the onset of bacteraemia and toxaemia. Therefore, vaccination probably remains the best prophylactic treatment.[1,2]

The disclosure of the structure of a tetrasaccharide **1**,[3] comprising a rare sugar called anthrose solely expressed by *B. anthracis* and related species,[4] has made it possible to investigate the development of glycoconjugate vaccines to replace or simply to complement the less than optimal currently licensed anthrax vaccines (Fig. 1).

Seeberger *et al.* were the first to synthesize the tetrasaccharide **1** and to confirm its importance as an immune target.[5] Further antigenicity/immunogenicity studies have established that the major antigenic determinants are expressed by the anthrose. Although the rhamnose units of **1** are not essential,[6–9] anti-anthrax spore recognition consistently increases from anthrose to the di/trisaccharide up to the tetrasaccharide.[6,7] Focusing on anthrose, the presence of a butanamido side-chain at C-4 seems to be mandatory.[7,10–12] Switching from a methyl to a hydroxymethyl group at C-6 leads to a complete loss of antigenicity,[8] apparently owing to steric clash rather than modification of an

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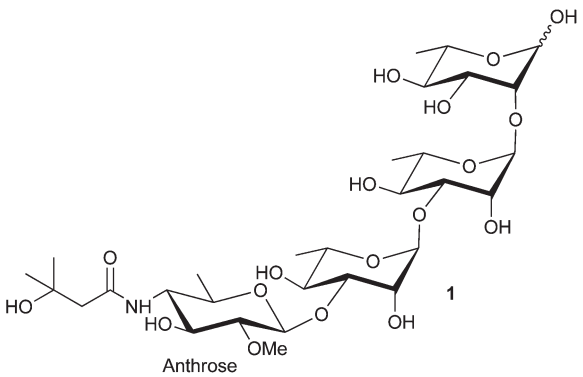
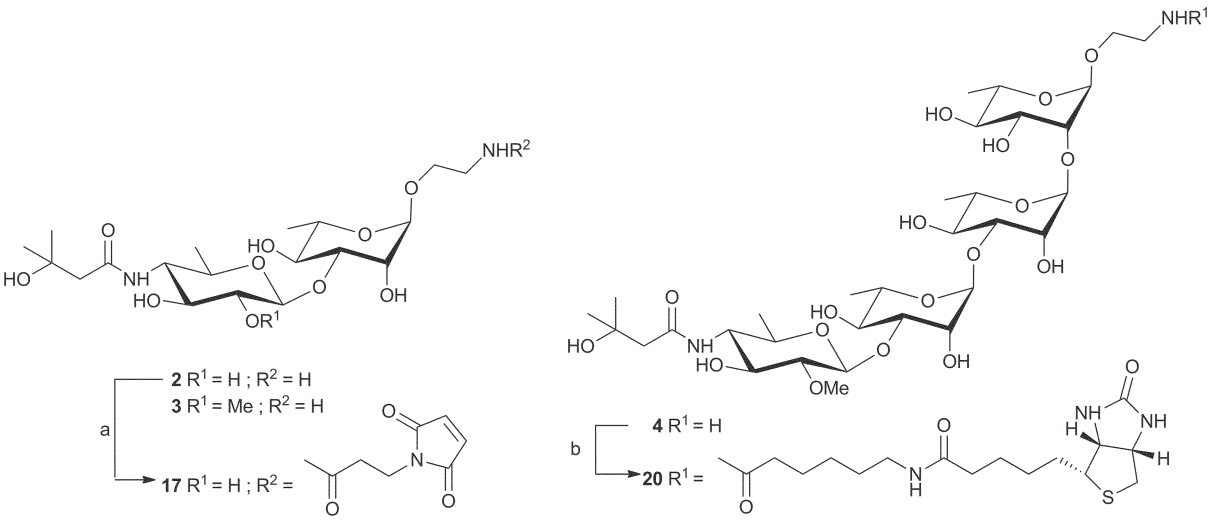


Fig. 1 Structure of the *B. anthracis* anthrose-terminated tetrasaccharide antigen **1**.



Scheme 1 Structures of di- and tetrasaccharide haptens related to the anthrax tetrasaccharide. *Reagents and conditions*: (a) 3-maleimidopropionic acid *N*-hydroxysuccinimide ester, DIPEA, DMF, rt, 1 h, 38%; (b) biotinamidohexanoic acid *N*-hydroxysuccinimide ester, DIPEA, CH_3CN, rt, 2 h, 86%.

important epitope as suggested by STD NMR experiments carried out with a monoclonal anti-tetrasaccharide antibody and a derivative related to **1**.[12] Noteworthy, replacement of the methoxy at C-2 by a hydroxyl group does not impair the recognition with anti-spores or anti-synthetic anthrax saccharide antibodies.[8,10] Moreover, serum induced in mice by conjugates made of *Shewanella* capsular polysaccharides, which contain a residue similar to anthrose but not methylated at O-2, have been shown to cross-react with *B. anthracis* spores, suggesting further that the methoxy at C-2 is not essential.[11] From this set of data, we have hypothesized that the truncated, demethylated anthropyranosyl-rhamnopyranoside derivative **2** would be a valuable mimic of the anthrax tetrasaccharide **1** while being more easily accessible (Scheme 1).

On the one hand, reducing the length of the hapten from a tetra- to a disaccharide would obviously result in a diminished number of synthetic steps. On the other hand, whatever the starting sugar or the strategy envisaged,[5,8,10,13–22] extensive protecting group manipulations are usually required to secure the introduction of the 2-methoxy as in anthrose. Besides, this functionalization must be carried out after the construction of the β-glycosidic bond which links anthrose to the ultimate rhamnose to avoid the formation of intractable α/β anomeric mixtures during glycosylation.[10,17]

We report herein the preparation of **2**, its conjugation to BSA used as a model protein carrier and the evaluation of the immune response induced in mice by the conjugates compared to that obtained by using the corresponding native disaccharide **3** (Scheme 1).

Results and discussion

Synthesis of the 2-*O*-demethylated-β-D-anthropyranosyl-(1→3)-α-L-rhamnopyranose disaccharide 2

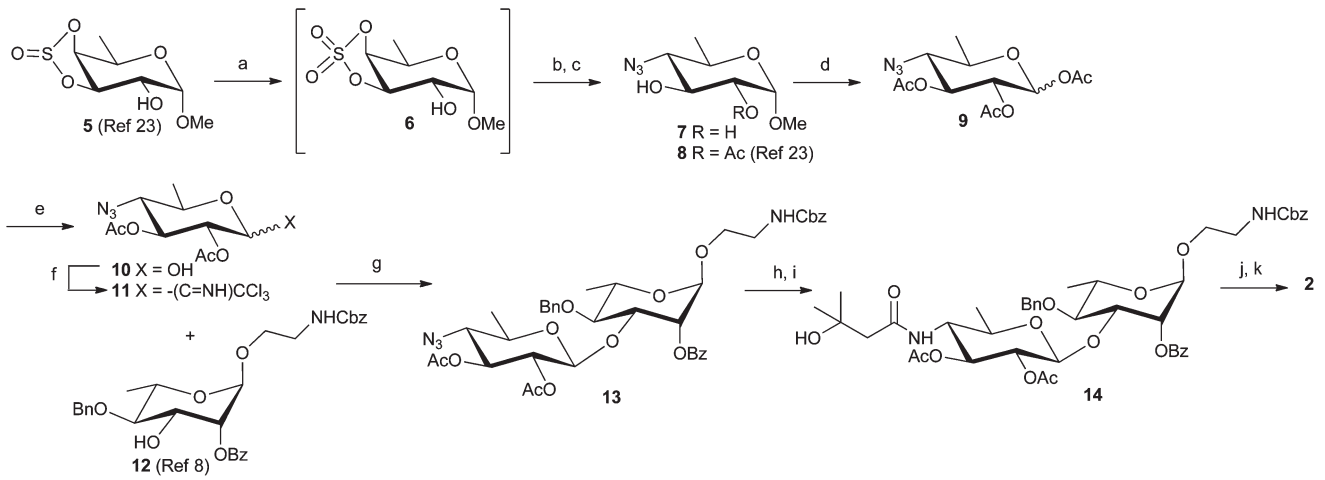
Preparation of disaccharide **2** could be envisaged from a glycosylation between a rhamnoside acceptor and an anthrose precursor donor. The latter could, in turn, advantageously be obtained starting from D-fucose, its synthesis mainly consisting of introducing selectively the amide side-chain at C-4 according to an S_N2-reaction. To this end, we decided to apply our recently disclosed strategy which makes use of a 3,4-cyclic sulphate D-fucopyranoside intermediate and which has led to the successful preparation of both spacer-equipped disaccharide **3** and anthrax tetrasaccharide **4** (Scheme 1).[23,24]

Thus, oxidation of known[23] cyclic sulphite **5** under Sharpless conditions gave rise to the corresponding cyclic sulphate **6** which was submitted to nucleophilic ring-opening using sodium azide followed by aqueous acid hydrolysis of the resulting acyclic sulphate to provide the 4-azido derivative **7** as the sole regioisomer (50% yield for the 3 steps) (Scheme 2). Alternatively, if **5** was first acetylated, 4-azido derivative **8**[23] was obtained in an improved 67% yield over the four steps. Further acetolysis[25] of **8** allowed the concomitant removal of the anomeric methyl group and protection of the remaining 3-OH to afford the tri-acetylated intermediate **9**. Selective deprotection of the anomeric acetate using benzylamine and activation with trichloroacetonitrile in the presence of DBU afforded glycoside donor **11** (66% yield for the three steps). This intermediate was thus obtained in 8 or 9 steps in 22 or 29% yield, respectively.

Glycosylation of donor **11** with known rhamnoside acceptor[8] **12** was next performed in the presence of TMSOTf to give disaccharide **13** in 80% yield. Reduction of the azide in **13** was carried out with sodium borohydride in the presence of nickel chloride[26] to provide an intermediate amine which was not isolated but further acylated to introduce the anthrose amide side-chain (55% for the two steps). Removal of the acetyl and benzoyl groups of intermediate **14** under Zemplén conditions followed by benzyl and Cbz-hydrogenolysis provided disaccharide **2** (82% yield for the two steps).

Preparation and characterization of the conjugates

Having the key disaccharide **2** and its native counterpart **3**[23] for comparison in hand, we next envisaged their conjugation to the



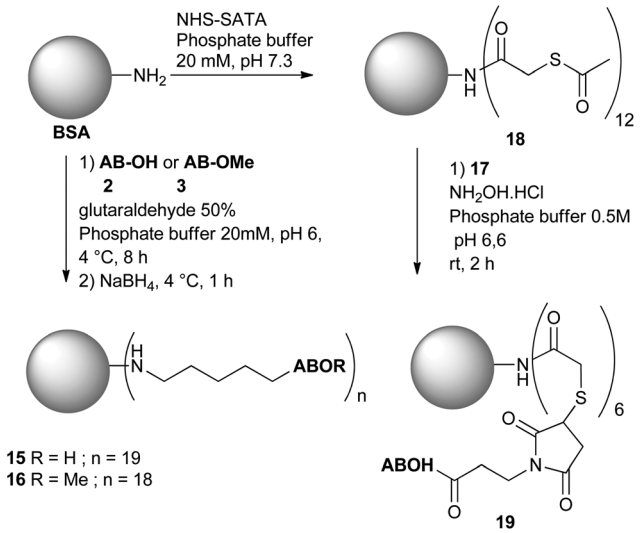
Scheme 2 Synthetic route for the preparation of **2**. *Reagents and conditions*: (a) $NaIO_4$, $RuCl_3{\cdot}H_2O$, CCl_4–CH_3CN–H_2O (1 : 1 : 1.5), 0 °C, 4 h; (b) NaN_3, DMF, 60 °C, 3 h; (c) H_2SO_4, H_2O, THF, rt, 1 h, 50% in three steps; (d) Ac_2O–AcOH–H_2SO_4, rt, 6 h; (e) $BnNH_2$, THF, rt, overnight; (f) CCl_3CN, DBU, CH_2Cl_2, 0 °C to rt, 2 h, 66% in three steps; (g) TMSOTf, 4 Å MS , CH_2Cl_2, −20 °C, 80%; (h) $NaBH_4$, $NiCl_2{\cdot}6H_2O$, EtOH–CH_2Cl_2, rt, 1 h; (i) 3-hydroxy-3-methylbutanoic acid, HATU, DIPEA, CH_2Cl_2, rt, 18 h, 55%; (j) MeONa (0.2 M in MeOH), rt, 12 h; (k) H_2 (10 bars), $Pd(OH)_2$/C, 50 °C, 30 min, 82% in two steps.

BSA protein carrier. Both disaccharides **2** and **3** were coupled to BSA using glutaraldehyde as a spacer *via* their respective free amino groups. The conjugates were further stabilized through reduction of the imine linkages to give conjugates **15** and **16** after dialysis (Scheme 3 and Table 1).

Alternatively, disaccharide **2** and BSA were derivatized using a maleimide and an acetylthio cross-linker to give intermediates **17** (Scheme 1) and **18** (Scheme 3), respectively. Disaccharide **17** and protein **18** were conjugated in the presence of an excess of hydroxylamine at pH 6.6 to afford the neoglycoconjugate **19** (Scheme 3 and Table 1).

We compared the MALDI-TOF mass spectra of the different preparations with that of BSA to determine their extent of derivatization (Table 1 and Fig. 2).

Similar molar sugar/protein ratios were found for conjugates **15** and **16** (19 and 18 mol mol^{-1}, respectively) while that of **19** was significantly lower (6 mol mol^{-1} on a maximum of 12 mol mol^{-1} considering the extent of derivatization of **18**). Total carbohydrate content of preparation **19** was also determined using high pH high-performance anion-exchange chromatography after TFA hydrolysis[28] of the conjugate. The amount of carbohydrate was very close to that estimated by mass spectrometry, suggesting that no or very little free sugar contaminant, known to be sometimes associated with hyporesponsiveness,[29,30] was present in vaccine preparation **19** (Table 1).



Scheme 3 Preparation of the conjugates.

Table 1 Characterization of the conjugates

Conjugate	Conjugation chemistry	Yield[a] (%)	Carbohydrate/protein mg/mg	µmol/µmol	Ratio
15	Glutaraldehyde	78	0.50/3.91	1.11/0.059	19
16	Glutaraldehyde	85	0.54/4.24	2.5/0.14	18
19	Thio/maleimide	57	0.22/3.99 (0.20 HPAEC-PAD)	0.36/0.06	6

[a] Yields were calculated on the basis of the weight of protein in the conjugate compared to the starting one determined by the Lowry method.[27]

Immunogenicity of the glycoconjugates

To assess the immunogenicity of conjugates **15**, **16** and **19**, groups of five female BALB/c mice were immunized subcutaneously (sc) using an equivalent of 10 µg of disaccharide per dose using the MPL® + TDM Adjuvant System (formerly known as RIBI®) from Sigma. Each group received three doses of the conjugates or phosphate buffered saline (PBS), used as a control, at three-week intervals. Anti-tetrasaccharide antibody (Ab) titers were determined by immobilizing biotinylated-tetrasaccharide **20**, obtained from **4** (Scheme 1), onto streptavidin-coated microtiter plates. PBS control sera contained low

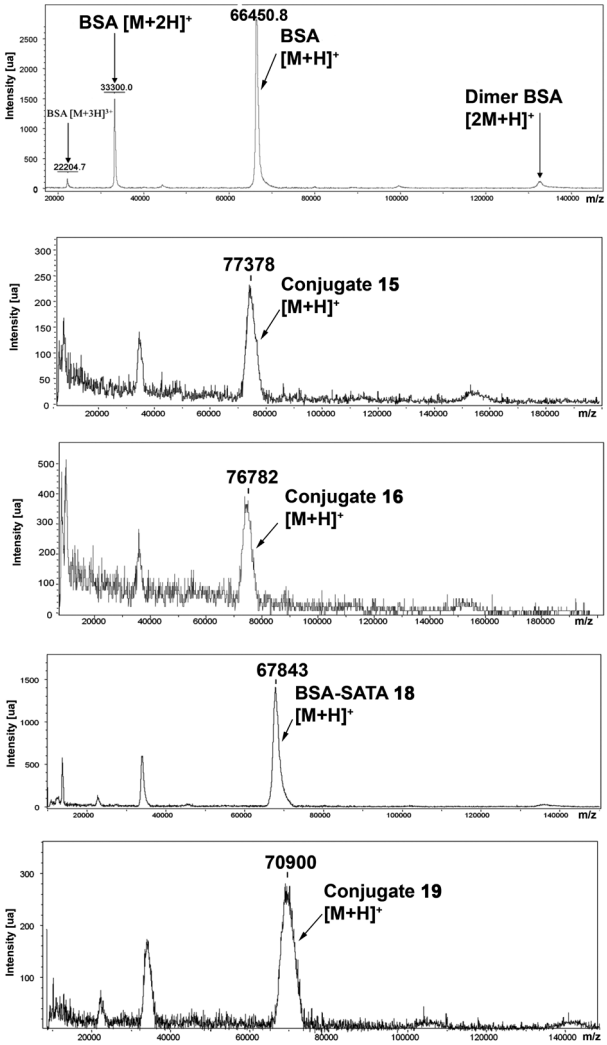


Fig. 2 Positive linear ion mode MALDI-TOF MS spectra of BSA (15 pmol) and of conjugates **15**, **16**, **18** and **19** (75 pmol). Mass spectra were generated by averaging the signal of 2000 single laser shots.

levels of anti-tetrasaccharide Abs. In contrast, all conjugates elicited anti-tetrasaccharide IgM and IgG Abs (Fig. 3).

Noteworthy, the mean IgG titres were maintained from the second to the third immunization (the apparent increase was not statistically significant). To further characterize the Ab response induced by the conjugates, IgG-subclasses Ab responses to the tetrasaccharide were measured in the sera obtained after the 3rd immunization (Fig. 4). Sub-typing indicated a bias towards a Th2 immune response as reflected by the prevalence of the IgG1 compared to the IgG2a,b response. A marked IgG3 response, typical of an anti-carbohydrate response, was also punctually observed. That conjugates of both native and 2-*O*-demethylated disaccharides related to the upstream moiety of **1** could induce a T-dependent immune response directed towards the anthrax tetrasaccharide is clearly established or at least confirmed for the former[5b] by these results. Previous antigenic[8,10,12] and immune[11] studies strongly supported that the presence of a methyl at *O*-2 of anthrose is not essential for the cross-reactivity. This new set of data suggests that **2** is equivalent to **3** as a hapten if one compares the IgG titers raised by the conjugates **15** and **16**. No statistical difference was observed between the Ab titers raised against conjugates **15** and **19**. However, further studies will be needed to determine whether the immune response is influenced by the conjugation chemistry since the two conjugates also differ by their extent of hapten loading. Importantly, the sera obtained after the third dose were shown to bind to *B. anthracis* spores suggesting that they recognize the anthrax tetrasaccharide as naturally expressed on exosporium (Fig. 5). These sera also cross-reacted with *B. cereus* spores, known to possess the anthrose biosynthetic operon[4] (data not shown).

Finally, whatever the conjugate tested, the mean titre was lower than that obtained when testing a hyperimmune serum used as a positive control, suggesting that further *B. anthracis* antigens should ideally be added to the carbohydrate hapten valency in a glycoconjugate immunogen against anthrax.

In conclusion, the data presented herein reinforce the potential use of synthetically accessible carbohydrate haptens to design a glycoconjugate vaccine active against anthrax. In our hands, demethylated disaccharide **2** proved to be as efficient an antigen as its native counterpart **3**, underlining that the 2-methyl group of anthrose is not required for immunogenicity.

Experimental section

General remarks

All reactions were monitored by TLC on Kieselgel 60 F254 (E. Merck). Detection was achieved by charring with vanillin. Silica gel (E. Merck, 240–400 mesh) was used for chromatography. Optical rotation was measured with a JASCO DIP-370 digital polarimeter, using a sodium lamp (λ = 589 nm) at 20 °C. All NMR experiments were performed at 300.13 Hz using a Bruker DMX300 spectrometer equipped with a Z-gradient unit for pulsed-field gradient spectroscopy. Assignments were performed by stepwise identification using COSY, successive RELAY, HSQC and HMBC experiments using standard pulse programs from the Bruker library. Chemical shifts are given relative to external TMS with calibration involving the residual solvent signals. For the chemical shift assignments of the disaccharides, A and B refer to protons or carbons of the anthrose or the rhamnose unit, respectively. Low-resolution ESI mass spectra were obtained on a hybrid quadrupole/time-of-flight (Q-TOF) instrument, equipped with a pneumatically assisted electrospray (Z-spray) ion source (Micromass). High-resolution mass spectra were recorded in positive mode on a ZabSpec TOF (Micromass, UK) tandem hybrid mass spectrometer with EBETOF geometry. The compounds were individually dissolved in MeOH at a concentration of 10 μg cm^{-3} and then infused into the electrospray ion source at a flow rate of 10 mm^3 min^{-1} at 60 °C. The mass spectrometer was operated at 4 kV whilst scanning the magnet in a typical range of 4000–100 Da. The mass spectra were collected as continuum profile data. Accurate mass measurement was achieved using polyethylene glycol as an internal reference with a resolving power set to a minimum of 10 000 (10% valley).

Protein concentrations were measured with the Lowry method[27] using BSA as a standard. Sugar/protein ratios were determined on an Autoflex III MALDI-TOF/TOF spectrometer (Bruker Daltonics) in positive ionization modes and with a linear

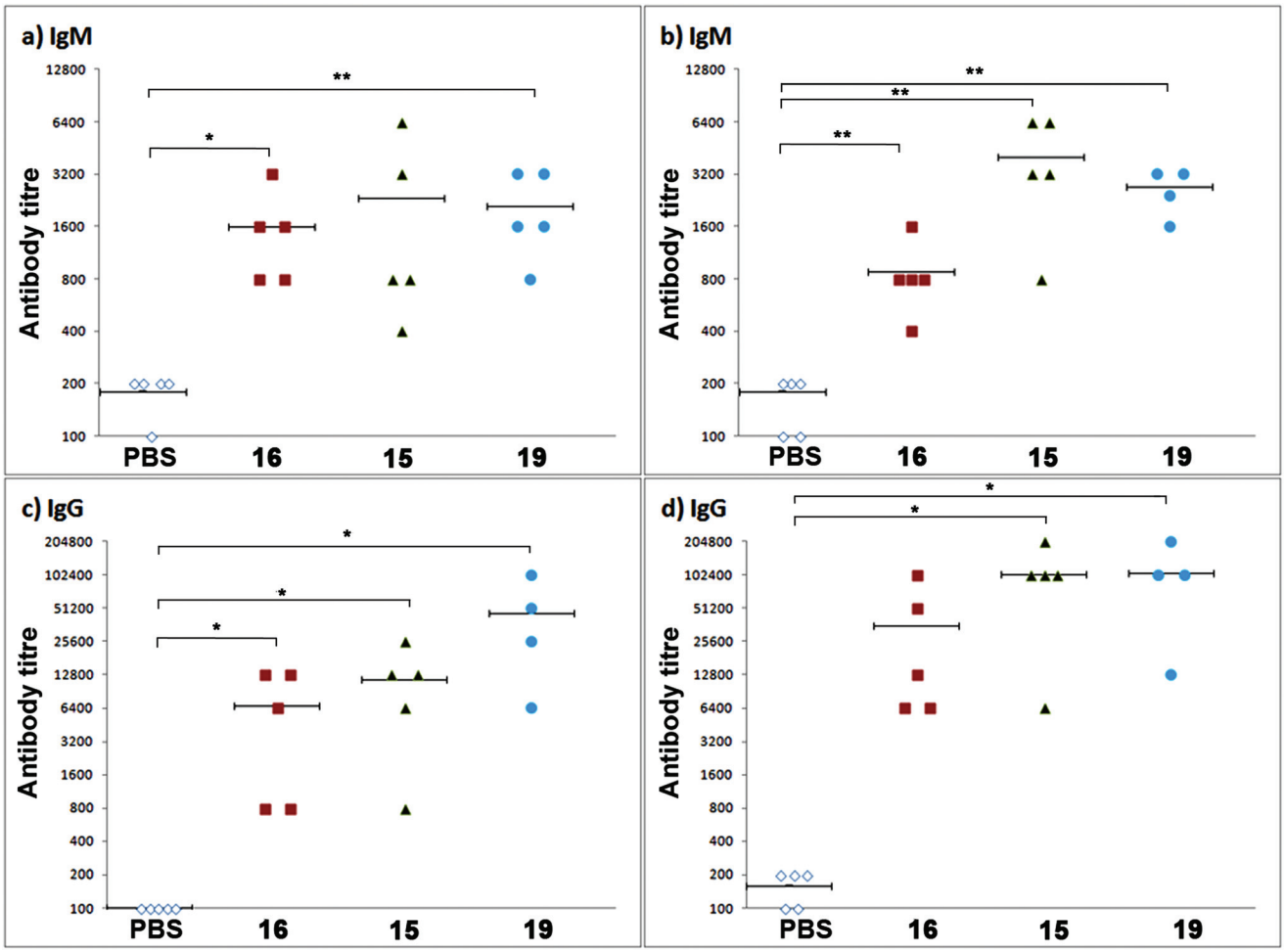


Fig. 3 ELISA anti-anthrax tetrasaccharide antibody titres after two (a) and (c) and three (b) and (d) immunizations with PBS (open rhombus) and conjugates **16** (square), **15** (triangle) and **19** (filled circle). ELISA plates were coated with biotinylated-derivative **20**. Each data point represents the titre for an individual mouse and the horizontal lines indicate the mean for the group of mice. A mouse of the group which had been immunized with conjugate **19** died between the 2nd and the 3rd immunization. Since no death was observed among the control group, toxicity of conjugate **19** cannot be ruled out. However, glycoconjugates are considered as safe and toxicity for anthrax tetrasaccharide-derived immunogens has not been reported previously. Moreover, the linker used to conjugate **2** to BSA is appropriate for use in humans.[31] Comparison of all results from the immunized groups with the control sera results were performed by ANOVA. *, $P < 0.05$; **, $P < 0.01$.

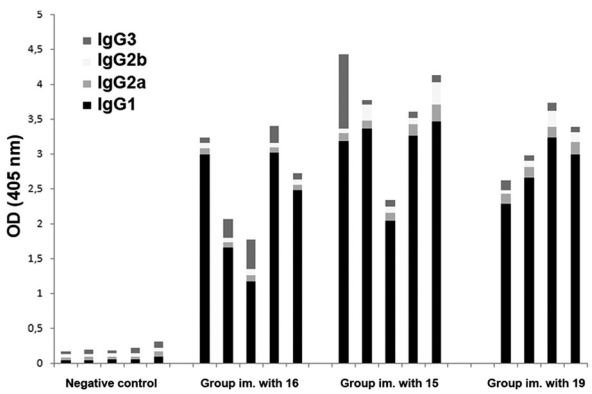


Fig. 4 Conjugates predominantly induce an anti-tetrasaccharide IgG1 response. IgG subclasses were analyzed by ELISA using **20** as coating agent (5 μg mL^{-1}) and sera collected after the third dose at a 1/100 dilution. Each bar represents the sum of the IgG1, IgG2a, IgG2b and IgG3 response for each individual from groups of mice immunized with PBS (control), conjugate **16**, **15** and **19**, respectively.

detection. 75 pmol of each samples were deposit on the MALDI target plate and were mixed with sinapinic acid as the matrix (10 mg mL^{-1}; H_2O–ACN–TFA, 50 : 50 : 0.1). The mass spectrometer was calibrated with a standard of BSA (15 pmol on target) on dimer and different charge states of the protein.

RP-HPLC purifications were performed on a Prevail C18 (10 × 250 mm, 5 μm) column, at a 3 mL min^{-1} flow rate and a gradient of 0% B during 5 min, 0 to 100% solvent B over 25 min, 100% B, 5 min (eluent A: 0.05% TFA in H_2O; eluent B: 0.05% TFA in CH_3CN–H_2O 60 : 40).

Methyl 4-azido-4,6-dideoxy-α-D-glucopyranoside (7)

To cyclic sulphite **5**[23] (877 mg, 3.92 mmol) was added a cold solution of CCl_4 (11.5 mL) and CH_3CN (11.5 mL). The mixture was cooled to 0 °C and cold water was added (17 mL). $RuCl_3 \cdot H_2O$ (13 mg, 0.06 mmol) and $NaIO_4$ (1.68 g, 7.84 mmol) were added in one portion and the reaction mixture was stirred

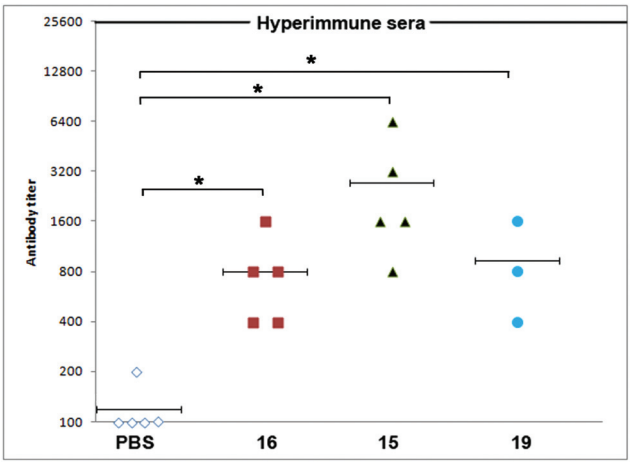


Fig. 5 Accessibility of *B. anthracis* spore surface-exposed anthrax tetrasaccharide by ELISA. Antibody titres after three immunizations with PBS (open rhombus) (negative control) and conjugates **16** (square), **15** (triangle) and **19** (circle). ELISA plates were coated with spores from *B. anthracis* A73 (Δ Ames) strain. Each data point represents the titre for an individual mouse and the horizontal lines indicate the mean for the group of mice. A positive control was provided by testing a hyper-immune serum (see Experimental section for details). The comparisons that are significantly different are PBS *versus* any group of conjugate-immunized mice (P value < 0.05).

vigorously at 0 °C. After 4 h stirring at this temperature, diethyl ether was added and the layers were separated. The aqueous layer was extracted with diethyl ether and the combined organic layers were washed with brine, dried over $MgSO_4$, filtered and concentrated under vacuum to give crude sulphate **6**. This material is used in the next step without any further purification.

A mixture of crude **6** (940 mg, 3.92 mmol) and NaN_3 (509 mg, 7.84 mmol) in dry DMF (5 mL) was stirred for 3 h at 60 °C. The solvent was then removed *in vacuo*. The residue was suspended in dry THF (5 mL) and concd H_2SO_4 (205 μL, 3.92 mmol) and water (72 μL, 3.92 mmol) were added and the mixture was stirred at room temperature for 1 h. The reaction mixture was then quenched with $NaHCO_3$ in excess and stirred for 20 min. The mixture was then concentrated under vacuum and the residue was purified by flash chromatography using cyclohexane–EtOAc (1 : 1) as eluent to give azide **7** (592 mg, 50% over 3 steps) as a white solid. R_f 0.24 (cyclohexane–EtOAc 1 : 1); $[\alpha]_D$ +154.5 (*c* 1, $CHCl_3$). ^1H NMR (300 MHz, $CDCl_3$) δ 4.75 (d, 1 H, $J_{1,2}$ = 3.9 Hz, H-1), 3.79 (t, 1 H, $J_{2,3}$ = 9.5 Hz, $J_{3,4}$ = 9.5 Hz, H-3), 3.64–3.56 (m, 2 H, H-2 and H-5), 3.43 (s, 3 H, CH_3), 3.07 (t, 1 H, $J_{4,5}$ = 9.5 Hz, H-4), 1.34 (d, 3 H, $J_{5,6}$ = 6.4 Hz, H-6); ^{13}C NMR (75 MHz, $CDCl_3$) δ 99.1 (C-1), 73.3 (C-3), 72.7 (C-2), 67.7 (C-4), 66.2 (C-5), 55.4 (CH_3), 18.2 (C-6); HR-ESIMS: *m*/*z* Calcd for $C_7H_{13}N_3O_4$ $[M + Na]^+$: 226.0804. Found 226.0800.

2,3-Di-*O*-acetyl-4-azido-4,6-dideoxy-D-glucopyranosyl trichloroacetimidate (11)

A solution of **8** (400 mg, 1.63 mmol) in Ac_2O–AcOH–H_2SO_4 (2.15 : 0.37 : 0.015 mL) was stirred at room temperature for 6 h and then was neutralized upon addition of $NaHCO_3$ at 0 °C. The reaction mixture was diluted with ethyl acetate and washed with water and brine. The organic layer was dried (Na_2SO_4), filtered and purified by flash chromatography using cyclohexane–EtOAc (7 : 3) as eluent to give tri-acetylated intermediate **9**. R_f 0.71 (cyclohexane–EtOAc 6 : 4); ESI-MS *m*/*z* 337.8 $[M + Na]^+$. Part of **9** (390 mg, 1.23 mmol) was further reacted with benzylamine (135 μL, 1.23 mmol) in dry THF (5 mL) at room temperature under inert atmosphere overnight and then quenched with aqueous 1 N HCl. The solvent was evaporated under reduced pressure and the crude residue was purified by flash chromatography using cyclohexane–ethyl acetate (6 : 4) as eluent to provide 2,3 di-*O*-acetyl-4-azido-4,6-dideoxy-D-glucopyranose **10** (247 mg, 73%) as a colorless oil. R_f 0.45/0.35 (cyclohexane : ethyl acetate 6 : 4); ESI-MS *m*/*z* 297.1 $[M + Na]^+$. To a solution of this intermediate (200 mg, 0.73 mmol) in CH_2Cl_2 (2 mL) were added trichloroacetonitrile (1.62 mL, 16 mmol) and DBU (25 μL, 0.16 mmol) dropwise at 0 °C. The mixture was then stirred at room temperature for 2 h and then concentrated *in vacuo*. The residue was purified by flash chromatography on silica gel using 2% Et_3N in cyclohexane–EtOAc (8 : 2) as eluent to give trichloroacetimidate **11** (274 mg, 90%) as a pale yellow oil as a mixture of α/β (>95/5) anomers. R_f 0.39 (cyclohexane–ethyl acetate 8 : 2); ^1H NMR (300 MHz, $CDCl_3$) δ 8.66 (s, 1 H, NHα), 6.46 (d, 1 H, $J_{1\alpha,2\alpha}$ = 3.5 Hz, H-1α), 5.54 (t, 1 H, $J_{2\alpha,3\alpha}$ = $J_{3\alpha,4\alpha}$ = 10.1 Hz, H-3α), 5.06 (dd, 1 H, $J_{1\alpha,2\alpha}$ = 3.5 Hz, $J_{2\alpha,3\alpha}$ = 10.1 Hz, H-2α), 3.99–3.87 (m, 1 H, H-5α), 3.34 (t, 1 H, $J_{4\alpha,5\alpha}$ = 10.1 Hz, H-4α), 3.34 (t, 1 H, $J_{4\alpha,5\alpha}$ = 9.5 Hz, H-4α), 2.13 (s, 3 H, CH_3), 2.01 (s, 3 H, CH_3), 1.39 (d, 3 H, $J_{5\alpha,6\alpha}$ = 6.2 Hz, H-6 α); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.0 (CO), 169.6 (CO), 160.9 (*C*=NH), 93.1 (C-1α), 77.6 (*C*Cl_3 α), 70.8 (C-2α and C-3α), 68.8 (C-5α), 65.5 (C-4α), 20.7 (CH_3), 20.5 (CH_3), 18.3 (C-6α); HR-ESIMS: *m*/*z* Calcd for $C_{12}H_{15}Cl_3N_4O_6$ $[M + Na]^+$: 438.9955. Found 438.9964.

2-[*N*-(Benzyloxycarbonyl)amino]ethyl (2,3-di-*O*-acetyl-4-azido-4,6-dideoxy-β-D-glucopyranosyl)-(1→3)-2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranoside (13)

Trimethylsilyl triflate (35 μL, 0.19 mmol) was added to a solution of donor **11** (200 mg, 0.48 mmol) and acceptor **12** (204 mg, 0.39 mmol) in CH_2Cl_2 (5 mL) containing 4 Å molecular sieves at −40 °C under an inert atmosphere. The temperature of the reaction mixture was raised to −20 °C over 1 h and then quenched upon addition of excess Et_3N. The crude mixture was filtered over a Celite® pad which was further washed with CH_2Cl_2. The filtrate was next evaporated under reduced pressure and the residue purified by flash chromatography on silica gel using cyclohexane–ethyl acetate (7 : 3) as eluent to give disaccharide **13** (241 mg, 80%). R_f 0.42 (cyclohexane–ethyl acetate 7 : 3); $[\alpha]_D$ −2.5 (*c* 1, $CHCl_3$). ^1H NMR (300 MHz, $CDCl_3$) δ 8.06 (br d, 2 H, *J* = 7.0 Hz, 2 H arom), 7.60 (br t, 1 H, *J* = 7.4 Hz, H arom), 7.51 (br t, 2 H, *J* = 7.4 Hz, H arom), 7.40–7.31 (m, 10 H, 10 H arom), 5.39 (dd, 1 H, $J_{1B,2B}$ = 1.8 Hz, $J_{2B,3B}$ = 3.4 Hz, H-2B), 5.22 (br t, *J* = 5.8 Hz, NH), 5.16 (s, 2 H, CH_2), 5.08 (t, 1 H, $J_{2A,3A}$ = $J_{3A,4A}$ = 9.6 Hz, H-3A), 4.95 (dd, 1 H, $J_{1A,2A}$ = 7.9 Hz, $J_{2A,3A}$ = 9.6 Hz, H-2A), 4.89 (s, 1 H, H-1B), 4.87 (d, 1 H, *J* = 11.0 Hz, A part of an AB system), 4.77 (d, 1 H, $J_{1A,2A}$ = 7.9 Hz, H-1A), 4.65 (d, 1 H, *J* = 11.0 Hz, B part

of an AB system), 4.21 (dd, 1 H, $J_{2B,3B}$ = 3.4 Hz, $J_{3B,4B}$ = 9.2 Hz, H-3B), 3.86–3.74 (m, 2 H, H-5B and C*H*H), 3.60 (t, 1 H, $J_{3B,4B}$ = $J_{4B,5B}$ = 9.9 Hz, H-4B), 3.64–3.55 (m, 1 H, CH*H*), 3.52–3.42 (m, 2 H, CH_2), 3.39–3.31 (m, 1 H, H-5A), 3.19 (t, 1 H, $J_{3A,4A}$ = $J_{4A,5A}$ = 9.6 Hz, H-4A), 2.07 (s, 3 H, CH_3), 1.77 (s, 3 H, CH_3), 1.32 (d, 3 H, $J_{5B,6B}$ = 6.3 Hz, H-6B), 1.25 (d, 3 H, $J_{5A,6A}$ = 6.1 Hz, H-6A); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.0 (CO), 169.7 (CO), 166.0 (CO), 156.4 (CONH), 138.0, 136.6, 133.1, 130.2, 129.9, 128.6, 128.5, 128.4, 128.2, 127.9, 127.8, 100.4 (C-1A), 97.3 (C-1B), 79.4 (C-4B), 78.8 (C3-B), 75.0 (CH_2), 74.0 (C-3A), 72.2 (C-2B and C-2A), 70.6 (C-5A), 67.8 (C-5B), 67.1 and 66.9 (2 CH_2), 65.4 (C-4A), 40.9 (CH_2), 20.6 (CH_3), 20.4 (CH_3), 18.0 (C-6A and C-6B); HR-ESIMS: *m*/*z* Calcd for $C_{40}H_{46}N_4O_{13}$ $[M + Na]^+$: 813.3018. Found 813.2938.

2-[*N*-(Benzyloxycarbonyl)amino]ethyl [2,3-di-*O*-acetyl-4-(3-hydroxy-3-methylbutanamido)-4,6-dideoxy-β-D-glucopyranosyl]-(1→3)-2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranoside (14)

To a solution of azide **13** (149 mg, 0.18 mmol) in a mixture of CH_2Cl_2 (2.8 mL) and EtOH (13 mL) was added $NaBH_4$ (14 mg, 0.38 mmol) and a catalytic amount of $NiCl_2{\cdot}6H_2O$. The reaction mixture was stirred at room temperature for 1 h and then was concentrated *in vacuo*. The residue was dissolved in CH_2Cl_2. The organic layer was washed with water and brine, dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude residue was used in the next step without purification. R_f 0.1 (cyclohexane–EtOAc 5 : 5); MS (ESI) *m*/*z* 787.1 $[M + Na]^+$.

To a solution of crude amine in dry DMF (10 mL) was added dropwise 3-hydroxy-3-methylbutanoic acid (45 µL, 0.36 mmol), followed by HATU (142 mg, 0.36 mmol), and then DIPEA (61 µL, 0.36 mmol). The reaction mixture was stirred under argon at room temperature for 18 h and was then diluted with CH_2Cl_2, washed with satd aq. $NaHCO_3$ and water. The organic phase was dried over Na_2SO_4, filtered and concentrated *in vacuo*. The residue was purified by flash-chromatography on silica gel using cyclohexane–EtOAc (6 : 4) as eluent to provide intermediate **14** (90 mg, 55% over 2 steps) as a colorless oil. R_f 0.14 (cyclohexane–ethyl acetate 3 : 2); $[\alpha]_D$ = −18.3 (*c* 0.5, $CHCl_3$); ^{1}H NMR (300 MHz, CD_3OD) δ 7.94 (br dd, 2 H, *J* = 7.2 Hz, *J* = 1.5 Hz, 2 H arom), 7.57–7.50 (m, 1 H, H arom), 7.41 (br t, 2 H, *J* = 7.6 Hz, H arom), 7.32–7.16 (m, 10 H, 10 H arom), 5.29 (br s, 1 H, H-2B), 5.06–4.92 (m, 2 H, H-3A and CH_2), 4.79–4.70 (m, 4 H, H-1A, H-2A, H-1B and A part of an AB system), 4.55 (d, 1 H, *J* = 11.2 Hz, B part of an AB system), 4.17 (dd, 1 H, $J_{2B,3B}$ = 3.5 Hz, $J_{3B,4B}$ = 9.5 Hz, H-3B), 3.77–3.37 (m, 6 H, H-4A, H-5A, H-4B, H-5B and CH_2), 3.31–3.23 (m, 2 H, CH_2), 2.16 (s, $CH_2C(CH_3)_2OH$), 1.81 (s, 3 H, CH_3), 1.58 (s, 3 H, CH_3), 1.14 (d, 3 H, $J_{5B,6B}$ = 6.2 Hz, H-6B), 1.10 (s, 6 H, 2 × CH_3), 0.98 (d, 3 H, $J_{5A,6A}$ = 6.0 Hz, H-6A); ^{13}C NMR (75 MHz, CD_3OD) δ 172.8 (CO), 170.5 (CO), 169.9 (CO), 166.1 (CO), 157.6 (CONH), 138.3, 136.6, 133.0, 129.4, 128.2, 128.1, 128.0, 127.6, 127.4, 100.4 (C-1A), 97.0 (C-1B), 79.2 (C-4B), 78.7 (C3-B), 74.4 (CH_2), 72.8, 72.6 and 72.5 (C-2A C-3A and C-2B), 70.5 (C-5A), 69.0 ($C(CH_3)_2OH$), 67.5 (C-5B), 66.3 and 66.1 (OCH_2 and CH_2Ph), 54.4 (C-4A), 48.1 (CH_2), 40.3 (NCH_2), 28.2 (2 CH_3), 19.4 (CH_3), 19.3 (CH_3), 17.0 and 16.6 (C-6A and C-6B); HR-ESIMS: *m*/*z* Calcd for $C_{45}H_{56}N_2O_{15}$ $[M + Na]^+$: 887.3578. Found 887.3589.

2-Aminoethyl 4-(3-hydroxy-3-methylbutanamido)-4,6-dideoxy-α-D-glucopyranosyl-(1→3)-α-L-rhamnopyranoside (2)

Compound **13** (67 mg, 0.08 mmol) was treated with NaOMe (0.2 M solution in MeOH) (800 µL, 0.16 mmol, 2 equiv.) for 12 h at rt. The reaction mixture was then neutralized by resin Amberlite IR120 H^+. The resin was filtered off and the filtrate was concentrated *in vacuo*. The crude product (54 mg, 0.08 mmol) was next treated without purification with a catalytic amount of a $Pd(OH)_2/C$ under 10 bars of hydrogen at 50 °C for 30 min in EtOH/AcOH (9 mL/1 mL). The reaction mixture was then concentrated *in vacuo*. The residue was dissolved in H_2O and was washed with CH_2Cl_2. The organic layer was extracted by water. The aqueous phases were combined and concentrated under reduced pressure. The crude was then purified by RP-HPLC to give **2** (30 mg, 82% over 2 steps). $[\alpha]_D$ = −7 ($CHCl_3$, *c* 0.5); ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.79 (br s, 1 H, H-1B), 4.64 (d, 1 H, $J_{1A,2A}$ = 7.7 Hz, H-1A), 4.13 (dd, 1 H, $J_{1B,2B}$ = 1.7 Hz, $J_{2B,3B}$ = 3.3 Hz, H-2B), 3.95–3.87 (m, 1 H, A part of an AB system, OC*H*H), 3.90 (dd, 1 H, $J_{2B,3B}$ = 3.3 Hz, $J_{3B,4B}$ = 9.2 Hz, H-3B), 3.69–3.54 (d, 5 H, H-4A, H-5A, H-4B, H-5B, B part of an AB system, OCH*H*), 3.47 (br t, 1 H, *J* = 9.4 Hz, H-3A), 3.35 (br t, 1 H, *J* = 8.4 Hz, H-2A), 3.27–3.15 (m, 2 H, CH_2NH), 2.41 (s, $NHCOCH_2$), 1.26 and 1.25 (2 × s, 2 CH_3), 1.24 (d, 3 H, $J_{5B,6B}$ = 5.5 Hz, H-6B), 1.18 (d, 3 H, $J_{5A,6A}$ = 5.7 Hz, H-6A); ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.1 (CO), 103.4 (C-1A), 99.7 (C-1B), 79.3 (C-3B), 74.0 (C-2A), 73.2 (C-3A), 71.2 and 70.9 (C-4B and C-5B), 70.2 ($C(CH_3)_2$), 69.6 (C-2B), 68.6 (C-5A), 66.3 (OCH_2), 56.5 (C-4A), 48.8 (CH_2), 39.0 (CH_2NH_2), 28.2 and 28.1 (2 CH_3), 17.1 (C-6B), 16.6 (C-6A); HR-ESI-MS *m*/*z* Calcd for $C_{19}H_{36}N_2O_{10}$ $[M + H]^+$ 453.2453, found 453.2428.

Conjugate 15

Disaccharide **2** (2 mg, 3.75 µmol, 50 equiv.) was added to a solution of BSA (5 mg, 0.075 µmol) in 20 mM potassium phosphate buffer solution, pH 6. The solution was cooled to 4 °C for 10 min and glutaraldehyde (10 µL, 50% in water) was added. The reaction mixture was kept at 4 °C for 8 h and then $NaBH_4$ was added. Following an additional period of 1 h at 4 °C, the reaction mixture was extensively dialyzed against 0.1 M phosphate buffer, pH 7.5 and then freeze-dried.

Conjugate 16

Disaccharide **3** (2 mg, 3.75 µmol, 50 equiv.) was added to a solution of BSA (5 mg, 0.075 µmol) in 20 mM potassium phosphate buffer solution, pH 6. The solution was cooled to 4 °C for 10 min and glutaraldehyde (10 µL, 50% in water) was added. The reaction mixture was kept at 4 °C for 8 h and then $NaBH_4$ was added. Following an additional period of 1 h at 4 °C, the reaction mixture was extensively dialyzed against 0.1 M phosphate buffer, pH 7.5 and then freeze dried.

2-[*N*-(Maleimidopropionyl)amido]ethyl 4-(3-hydroxy-3-methylbutanamido)-4,6-dideoxy-α-D-glucopyranosyl-(1→3)-α-L-rhamnopyranoside (17)

To a solution of disaccharide **2** (12 mg, 26 μmol) in DMF (500 μL) were added 3-maleimidopropionic acid *N*-hydroxysuccinimide ester (7 mg, 26 μmol, 1 equiv.) and DIPEA (9 μL, 52 μmol, 2 equiv.). The progress of the reaction was monitored by HPLC. After 1 h at room temperature, the reaction was complete. The crude was then purified by RP-HPLC to give **17** (6 mg, 38%) as a white solid after freeze-drying. ESI-MS *m*/*z* 625.9 $[M + Na]^+$.

SATA-activated BSA 18

To a solution of BSA (7 mg, 0.105 μmol) dissolved in PBS 20 mM, pH 7.3 (700 μL), was added NHS-SATA (3 × 0.61 mg, 3 × 2.10 μmol, 3 × 20 equiv., dissolved in 30 μL of CH_3CN) in three portions every 45 min. The pH of the reaction mixture was controlled (indicator paper) and maintained at 7–7.5 by addition of 0.5 M aqueous NaOH. Following an additional reaction period of 40 min, the crude reaction mixture was extensively dialyzed against 0.02 M potassium phosphate buffer, pH 6.6 at 4 °C.

Conjugate 19

SATA-activated **18** in 20 mM potassium phosphate buffer solution was reacted with disaccharide **2** (1 mg, 1.60 μmol, 15.3 equiv.). Reaction mixture was buffered at a 0.5 M concentration by addition of 1 M potassium phosphate buffer, pH 6.6. Then $NH_2OH{\cdot}HCl$ (7.5 μl of a 2 M solution in 1 M potassium phosphate buffer, pH 6.6) was added to the mixture and the coupling was conducted for 2 h at room temperature. The conjugated product was purified by dialysis against 0.1 M phosphate buffer, pH 7.5, and freeze-dried.

Sugar content was determined both by MALDI-MS and by high-performance anion-exchange chromatography following TFA hydrolysis of the conjugate using disaccharide **3** as a standard[28] as follows:

50 μL of sample was mixed with 12.5 μL of 10 N TFA (final concentration 2 N) in a glass tube sealed with a screw-cap. After 2 h at 100 °C, solutions were freeze-dried and then re-dissolved in 50 μl of water and the solutions transferred to autosampler vials. In separate tubes, standard mixtures of known amounts of disaccharide **3** (from 0 to 50 μg) were treated similarly and were used for chemical identification and quantification. Chromatography of the samples was performed using a Dionex ICS 3000 system and a pulsed amperometric detector. 10 μL of the extract were injected through a 4 × 50 mm Propac PA1 pre-column (Dionex), before separation of anionic compounds on a 4 × 250 mm Propac PA1 column (Dionex) at 35 °C, at a flow rate of 1 ml min^{-1}. Peak analysis was performed using Chromeleon software, version 7.0. Solvent A was H_2O; solvent B was a 100 mM NaOH solution; and solvent C was a 100 mM NaOH containing 0.5 M sodium acetate solution. Anionic compounds were eluted with a multi-step gradient as follows: 0–7 min, 20 mM NaOH; 7–15 min, 20 to 100 mM NaOH; 15–60 min, 100 to 0 mM NaOH and simultaneous gradient of NaAc from 0 to 500 mM. After every run, the column was re-equilibrated in 20 mM NaOH for 10 min. Analyses were carried out in triplicates.

2-[*N*-(Biotinamidohexanoyl)amido]ethyl [4-(3-hydroxy-3-methylbutanamido)-4,6-dideoxy-2-*O*-methyl-β-D-glucopyranosyl]-(1→3)-α-L-rhamnopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranoside (20)

To a solution of tetrasaccharide **4** (3 mg, 3.4 μmol) in acetonitrile (100 μL) were added biotinamidohexanoic acid *N*-succinimidyl ester (1.6 mg, 3.4 μmol, 1 equiv.) and DIPEA (0.9 μL, 5.2 μmol, 1.5 equiv.). The reaction mixture was stirred for 2 h at room temperature and then concentrated *in vacuo*. The crude was then purified by RP-HPLC to give **20** (3 mg, 86%) as a white solid after lyophilisation. ESI-MS *m*/*z* 1120.6 $[M + Na]^+$.

Immunizations

Groups of five 8-week-old female BALB/c mice were injected subcutaneously three times at 3-week intervals with 10 μg of conjugate, in saline solutions containing Sigma Adjuvant System® (reference: S6322). The negative controlled group of mice was immunized similarly with PBS. Mice were bled 1 week after the second and the third immunizations. The IgM and IgG Ab response was measured by ELISA against the biotinylated tetrasaccharide **20** immobilized on streptavidin-coated microtiter plates as previously described.[32,33] The Ab titre was defined as the dilution of immune serum that gave an OD (405 nm) at least twice that observed with pre-immune serum.

Anti-spore ELISA

If not stated otherwise, all incubation steps were carried out at room temperature, alike all washing steps were carried out using PBS containing 0.05% Tween 20. *B. anthracis* spores (A73) were diluted in a 0.1 M carbonate buffer at a concentration of 10^8 spores per ml. Each well of a 96-well high binding microtiter plate (Nunc, Roskilde, Denmark) was coated with 100 μl of the prepared coating solution and incubated overnight at 4 °C. Thereafter the wells were washed twice and blocked with 10% (v/v) FCS in PBS for 1 h. Duplicates of the serum samples were serial diluted in log2-steps in blocking solution on the plate at a final volume of 100 μl per well and incubated for 2 h. As controls a known positive serum (see below) (single serial dilution) and a negative serum (single point measurement) and at least 6 blanks only containing blocking solution were included. After washing the plates 5 times 100 μl of prepared secondary antibody solution in blocking solution was added to the wells and incubated for 1 h. Antigen-specific antibodies used were polyclonal goat anti-mouse IgG (Sigma-Aldrich, St. Louis, MO) conjugated to horseradish peroxidase. Plates were washed again 6 times and developed with 100 μl per well of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) substrate (Roche Diagnostics, Manheim, Germany) and incubated for 30 min in the dark. Absorbance was measured at 414 nm in a microtiter plate reader (Model 680, Biorad Laboratories, Hercules, CA). Endpoint titers were defined as the

reciprocal of the highest serum dilution that resulted in an absorbance greater than an OD value of 0.1.

As a positive control, a hyperimmune sera prepared as follows was used: Briefly, 10 NMRI mice were immunized with a mixture of rBclA, rPA83, capsule-conjugate and formaldehyde-inactivated spores including lipopeptide as an adjuvant. They were immunized three times at two week intervals and were then challenged with a 25 × LD_{50} of a virulent Ames strain. Three weeks after the challenge the survivors were bled and killed. The pooled sera of the survivors were used as the positive control.

Statistical analysis

Statistical comparisons between experimental groups were performed using one-way analysis of variance (ANOVA) and Tukey's post-test comparison. The results were significant if the difference between the analysed groups equalled or exceeded the 95% confidence level ($P < 0.05$). Statistics were performed with ORIGIN 7.5 PRO software (OriginLab).

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