Synthetic methods to glycerol teichoic acids

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Chapter 6

Synthesis of TA-Protein Conjugates and Their Immunological Evaluation

Introduction

Many Gram positive bacteria, such as *Staphylococcus aureus, Enterococcus faecalis, Streptococcus pneumonia* and *Clostridium difficile*, pose an emerging risk in healthcare due to the increasing resistance against antibiotics.[1] *E. faecalis* has become second in line of most common nosocomial infections and this normally harmless commensal poses a considerable threat to immunocompromized and critically ill patients as the source of infections such as bacteremia, peritonitis, endocarditis, urinary tract and wound infections.[2] As a result there is a growing interest in the development of alternative treatments and preventive strategies to combat these bacteria. With the identification of several virulence factors of *E. faecalis,* the possibility for the development of prophylactic and therapeutic vaccine strategies becomes an attractive option.[3]

Several virulence factors involved in the pathogenesis of enterococcal infections have been identified, including cell surface exposed proteins, involved in the adherence of the bacteria to extracellular structures, biofilm formation and colonization events, and cell-wall carbohydrate antigens, such as capsular polysaccharides (CPs) and lipoteichoic acids (LTAs).[4] In 2006, Theilacker *et al.* demonstrated that non-encapsulated strains of *E. faecalis*, which account for about half of the clinical isolates, are opsonized by rabbit antibodies raised against purified LTA derived from these bacteria.[5] Passive immunization of infected mice with this antiserum led to clearance of *E. faecalis* and *S. epidermis* in the bloodstream. Additionally, it was revealed that the rabbit antiserum raised against *E. faecalis* LTA opsonized a selection of clinically relevant enterococcal, streptococcal and staphylococcal strains in an opsonophagocytic killing assay (OPA). These results indicate that the opsonic antibodies were raised against the common non-substituted 1,3-glycerol phosphate LTA-backbone. With the aid of synthetic TA oligomers, described in **chapter 3** and **chapter 5** of this thesis, it was shown that relatively short non-substituted oligoglycerol phosphates were capable of inhibiting killing by opsonic antibodies raised against native *E. faecalis* LTA.[6] Using the same opsonophagocytic killing inhibition assay (OPIA) it was revealed that the incorporation of a single α-

glucosyl substituent in a TA hexamer fragments led to a significantly improved inhibitor, indicating that the glucosylated oligomers could function as potent antigens.[7] Notably, this type of substitution does not occur in *E. faecalis* LTA. The identification of TA hexamer **1** (Figure 1) as a possible synthetic antigen invites the use of this compound in a model vaccine for passive and/or active immunization strategies. This chapter describes the development of a semi-synthetic TA-carrier protein conjugate as a possible vaccine candidate.

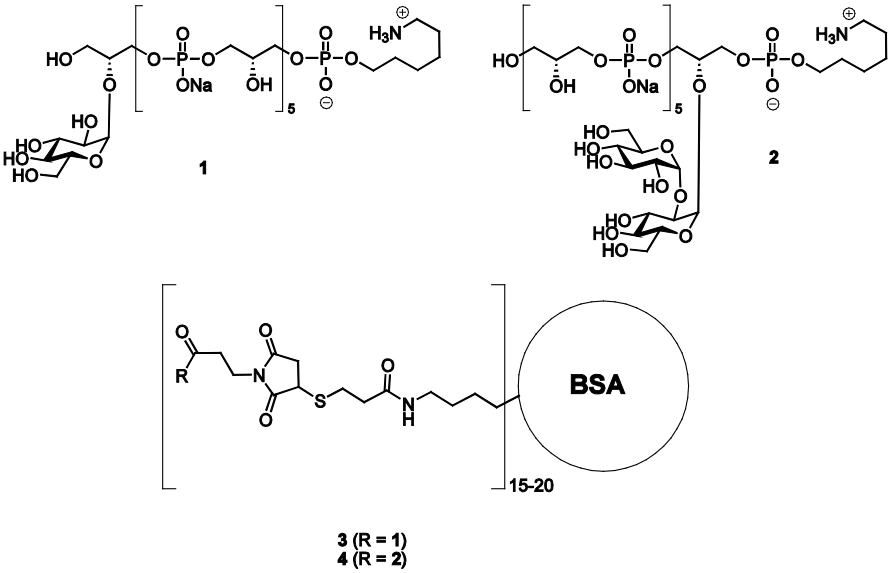


Figure 1. Active **1** and inactive **2** and their conjugates with BSA (**3**,**4**).

Results and Discussion

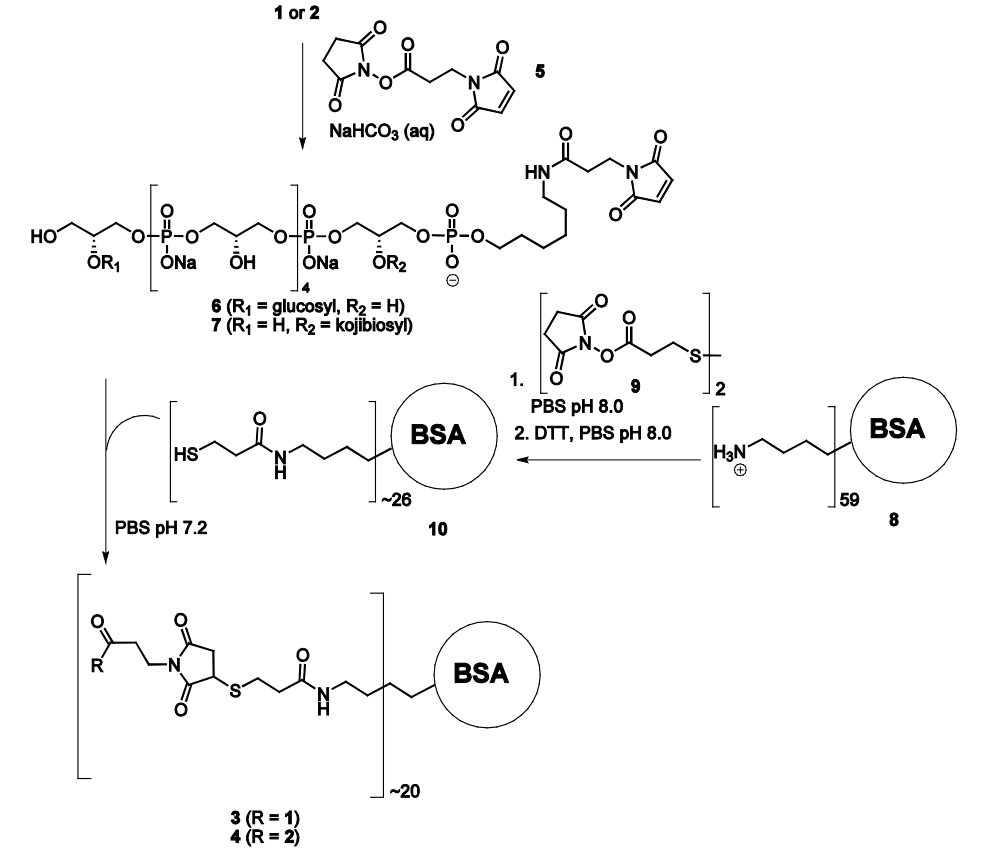
Small (carbohydrate) antigens, such as fragment **1**, are generally poorly immunogenic and only induce a T-cell independent immune response. A well-established approach to improve this low immune response comprises the conjugation of the antigen to an immunogenic carrier protein. This leads to T-cell dependent immune response, invoking immunological memory, affinity maturation, and IgM to IgG isotype switching.[8-10] Conjugate vaccines[11-17] are capable of eliciting an immune response in infants below the age of two years and this type of vaccines is now commonly used in broad national vaccination programs worldwide. A notable example of a semi-synthetic glycoconjugate vaccine that is currently used in Cuba and Vietnam and other countries is represented by Quimihib®, a vaccine developed against *Haemophilus influenza Type b* by Verez-Bencomo *et al.* comprising a fully synthetic antigen covalently linked to a tetanus toxoid (TTd) carrier protein.[17] Various (semi)-synthetic conjugate vaccines against important pathogens and specific cancer types are currently under development.[8,9,18]

For the development of conjugate vaccines several proteins can be used as immunogenic carrier, such as the tetanus toxoid mentioned above, diphteria toxoid (DTd), CRM197 (a non-toxic recombinant form of DTd), keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Because of its stability, ease of handling and modification possibilities, the latter protein was selected as a carrier in the first experimental vaccine. Its favorable molecular weight (~67 kD) as well as its non-glycosylated nature allows the use of several analytical techniques to characterize the conjugate.[10]

Two synthetic antigens were selected for conjugation to BSA: TA hexamer **1**, featuring a glycosyl substituent on the terminal glycerol phosphate moiety and kojibiosyl-containing fragment **2** (Figure 1). The former was selected because it represent the most active synthetic fragment from the assembled TA-oligomer library, as indicated by the OPIA described in **chapter 3**. The latter structure was used, because this structure, although it includes the natural glycosyl substituent found in *E. faecalis* LTA, showed a relatively poor inhibitory activity and therefore the projected conjugate serves as a relevant control compound. The conjugation of haptens to carrier proteins such as BSA can be accomplished with the aid of various chemical strategies and the aminohexyl functionality present on TA fragments **1** and **2** allows the installment of a conjugation handle of choice. It was decided to adapt the conjugation strategy used by Verez-Bencomo *et al.* in the development of the Quimihib®-vaccine, because of the structural similarities between the Hib capsular polysaccharide and the TA-oligomers. In this procedure the phosphodiester glycan fragments are equipped with a maleimide functionality and the lysine side chain amino functions in the protein are decorated with thiopropionyl moieties to allow for a robust and reliable conjugation reaction through a Michael type addition.[17,19]

As depicted in Scheme 1, treatment of **1** and **2** with the hydroxysuccinimide ester of 3-maleimidopropionic acid (**5**) in a basic mixture of DMF and H_2O gave, after size exclusion chromatography, the maleimides **6** and **7** in 67 and 86% yield, respectively. BSA (**8**) was treated with the homodisulfide of 3-thiopropionic acid hydroxysuccinimide ester (**9**) in a mixture of DMF and phosphate buffer saline (PBS, pH 8.0). After reduction of the disulfide bonds with dithiothreitol (DTT) and dialysis under inert atmosphere, the thiolated BSA (**10**) was obtained.[19] Quantification of the thiols present on the protein was performed by reaction of **10** with Ellman's reagent followed by UV_{412} analysis, indicating the presence of an additional ~26 thiol functionalities (total amount of thiol groups ~43) compared to DTT treated BSA (**8**, ~17 thiols per protein). Maleimides **6** and **7** were conjugated with thiolated-BSA **10** (1.2 eq of maleimide with respect to the total amount of thiol groups were used) to give conjugates **3** and **4**, which were purified by dialysis and analyzed on protein and sugar content. Based on UV_{280} spectrometry and phenol/H_2SO_4 colorimetric assays a sugar:protein ratio of ~1.5:1 was established for both conjugates. SDS-PAGE analysis of the conjugates showed broad bands around ~95 kDa and ~100 kDa, respectively,

Scheme 1. Synthesis of conjugates **3** and **4**.



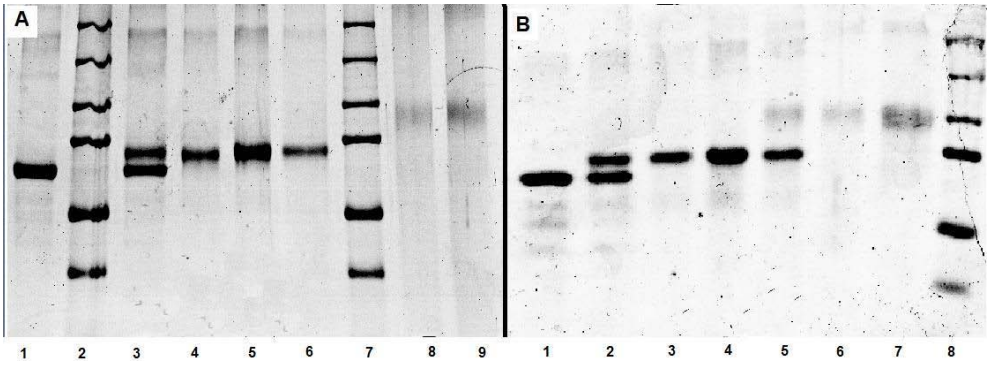


Figure 2. SDS-PAGE followed by Coomassie Brilliant Blue staining of conjugates **3** (A) and **4** (B). A: BSA (lane 1,3), **10** (lane 3-6), conjugate **3** (lane 8,9), DC-marker (lane 2,7; top to bottom: 250, 150, 100, 75, 50, 37 kDa). B: BSA (lane 1,2), **10** (lane 2-5), conjugate **4** (lane 5-7), DC-marker (lane 8).

indicating protein:sugar ratio's of ~2.4:1 and 2.0:1 for 3 and 4, respectively. These data suggest a substitution rate of around 20 (+/- 5) TA-fragments per BSA (see Figure 2).

To evaluate the immunological activity, rabbits were repeatedly immunized s.c. and subsequently i.v. with conjugate **3**, **4** or native BSA. After five weeks, serum was obtained and tested regarding the presence of opsonic antibodies against *E. faecalis* in an opsonophagocytic killing assay (OPA). In this assay appropriate serum dilutions (100 µl) were added to a mixture of human leukocytes (100 µl, 2 x 10^7 cells/ml), complement proteins (preadsorped baby rabbit serum, 100 µl) and *E. faecalis* (100 µl, 2 x 10^7 CFU/ml). After incubating 90 minutes at 37 °C on a rotor rack, the remaining number of viable enterococcal colony forming units (CFUs) was quantified. The results of this OPA are depicted in Figure 3, where it can be seen that only the serum raised against conjugate **3** was able to significantly kill *E. faecalis*. Anti-**4** serum gave rise to a low amount of opsonic antibodies and the anti-BSA serum showed no opsonic killing at all. These results show that fragment **1** not only effectively binds to opsonic antibodies raised against native enterococcal LTA, as revealed in **chapter 3**, but that it can also be used as an immunogen when present as a BSA conjugate *in vivo*. As a control experiment an opsonophagocytic inhibition assay (OPIA) was performed with anti-**3** serum on *E. faecalis* and *E. faecium* using conjugate **3** as an inhibitor. Figure 4 demonstrates the specificity of the opsonic killing observed. While serum dilutions of 1:1,000 were effectively able to kill *E. faecalis* and *E. faecium*, this killing activity could be removed by absorbtion with conjugate **3** in a dose-dependent fashion.

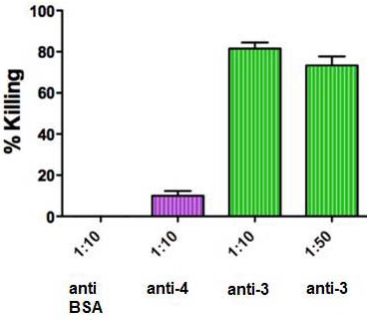


Figure 3. Opsonic killing of *E. faecalis* by rabbit antiserum raised to conjugates **3** and **4**. Anti-BSA is used as a control (left).

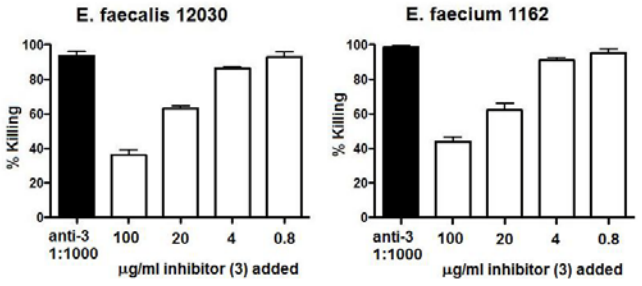


Figure 4. OPIA assay of different amounts of construct **3** using anti-**3** serum against *E. faecium* and *E. faecalis*. The black bars on the left represent the killing of the bacterium by anti-**3** in the OPA when no inhibitor is present (negative control).

As described above, Theilacker *et al.* observed that serum raised against native enterococcal LTA showed cross-reactivity towards a selection of Gram-positive species.[6] Therefore, it was explored whether the anti-**3** rabbit serum was able to

opsonize some of these bacteria. To this end, the previously mentioned OPA was performed using strains of *Enterococcus faecalis*, *Enterococcus faecium* and *Staphylococcus aureus*. As revealed in Figure 5, *E. faecalis* is readily opsonized by the serum. The closely related *E. faecium* was effectively killed in the OPA and also *S. aureus* was opsonized by anti-**3** serum to a significant extent (i.e. about 70% killing at a serum dilution of 1:1,000). Because the OPA showed that the serum raised against conjugate **3** could be diluted 5000-fold with minimal loss of activity against *E. faecalis* and *E. faecium* the serum was investigated at higher dilutions (Figure 6). This revealed that *E. faecalis* is significantly opsonized by this serum up to a 20.000-fold dilution (~70 % killing). Notably, the serum proved even more potent against *E. faecium* and could be diluted 160.000 fold before a significant drop in activity was observed.

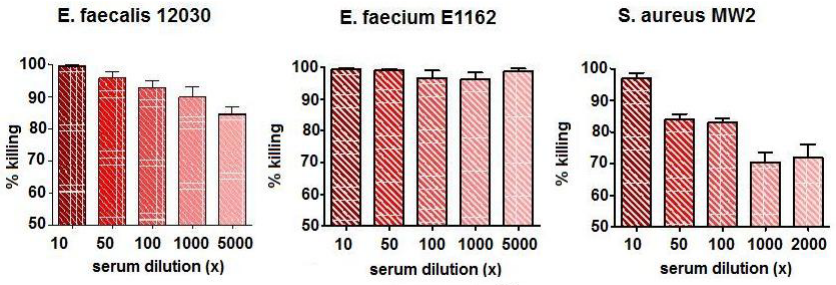


Figure 5. Crossreactivity of the serum: Opsonic killing of *E.Faecalis*, *E. Faecium* and *S. Aureus* by rabbit serum (dilutions 1:10 ➔ 1:5000) raised against conjugate **3**.

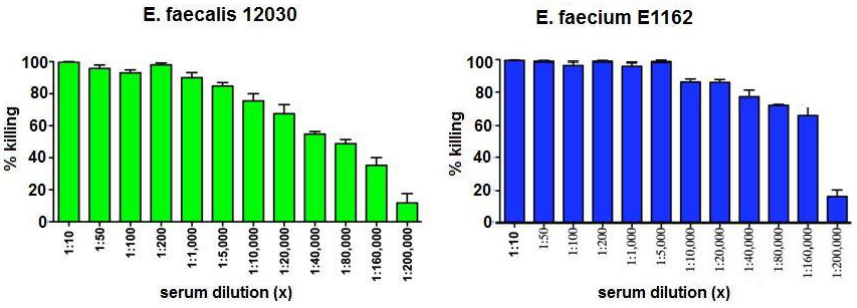


Figure 6. Opsonic killing of *E. Faecalis* (left) and *E.Faecium* (right) by rabbit anti-**3** at dilutions up to 1:200.000.

The next step of the immunological evaluation of conjugate **3** comprised an *in vivo* passive immunization experiment using the serum raised against this conjugate. To this end, a modification of a rat endocarditis model, described originally by Lee *et al.* to measure the protective properties of antiserum raised against *S. aureus* type 5 capsular polysaccharide, was used.[20] Rats were treated with normal rabbit serum (NRS) or anti-**3** serum 48 and 24h before they were challenged intravenously with *E. faecalis*. Four days after the infection, rats were euthanized and the total weight of bacterial vegetation on the aortic valve determined. In addition, the amount of colony

forming units (CFUs) per mg of the harvested vegetation was determined. It was found that the weight of endocarditic vegetation present on the aortic valves of the rats receiving NRS was between 1 and 3 mg (see Figure 7A). The total weight of vegetations found on the valves of rats immunized with the anti-**3** serum was significantly less (~0.5 mg). The potency of the serum was also revealed by the significant reduction of CFUs present in the bacterial vegetation: 10^4-10^7 CFUs per mg vegetation were present in rats receiving NRS, whereas bacterial vegetations in the anti-**3** immunized rats contained 10^1-10^4 CFUs per mg on average (see Figure 7B).[21]

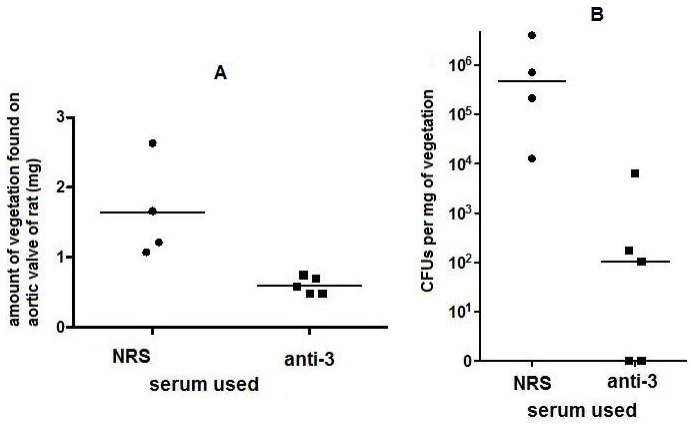


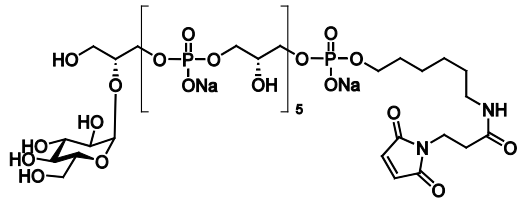
Figure 7. Effects of anti-**3** serum in a rat endocarditis model. A: Bacterial matter (in mg) found on the murine aortic valves immunized with either NRS or anti-**3** serum. B: CFUs found in the corresponding vegetation (CFUs/mg).

Conclusion

Carbohydrate substituted glycerol TA hexamers **1** and **2** were successfully attached to BSA (**8**) using thiol-maleimide chemistry resulting in conjugates **3** and **4**, respectively. Conjugate **3** showed promising results in initial immunological evaluation experiments and it was shown subsequently that rabbit serum raised against conjugate **3** was able to kill *E. faecalis* in an OPA. In addition, the serum was able to opsonize individual bacterial strains of two other Gram-positive species (*S. aureus* and *E. faecium*). Notably, the activity of the serum against *E. faecium* was especially high and it was shown that the serum could be diluted 160.000-fold before a significant drop in activity resulted. Passive immunization with the rabbit anti-**3** serum protected rats against *E. faecalis* in an endocarditis model. The initial immunological evaluation of conjugate **3** indicates that it is a promising lead candidate for a semi-synthetic vaccine against *E. faecalis* and possibly also as a broad-spectrum vaccine directed against several other Gram-positive species. Notably, the results described here show that a relatively small hexameric fragment **1** can be used to elicit an immune response against native LTA. This bodes well for the future development and large scale production of a semi-synthetic synthetic TA vaccine. The next step in the evaluation of conjugate **3** will comprise its evaluation in an active immunization setting.

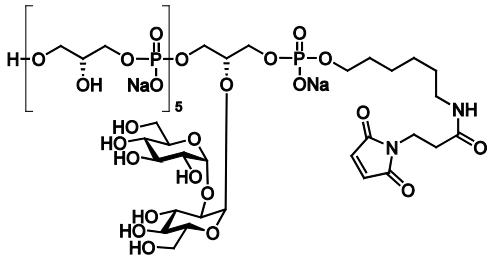
Experimental section

General procedures: ^{31}P, ^{1}H, and ^{13}C NMR spectra were recorded with a Bruker AV 400 (161.7, 400 and 125 MHz respectively) or a Bruker DMX 600 (600 and 150 MHz respectively). ^{1}H-NMR spectra were recorded in D_2O with chemical shift (δ) relative to HDO (4.755 ppm) at room temperature. ^{31}P spectra were measured with chemical shift relative to 85% H_3PO_4 (external standard) and ^{13}C-NMR spectra were recorded with chemical shift relative to TMS (external standard). High resolution mass spectra (HRMS) were recorded by direct injection (2 µl of a 2 µM solution in water/acetonitrile; 50/50; v/v and 10mM ammonium formate) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 275 °C) with resolution R = 60000 at m/z 400 (mass range m/z = 150-2000) and dioctylphthalate (m/z = 391.28428) as a lock mass. The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). Analysis of the conjugates (**3**, **4**) comprised of sugar quantification[22] (phenol/sulfuric acid assay, using TAs **1** and **2**, respectively, as external standards), protein quantification (UV_{280} absorption, using BSA as an external standard) and SDS-PAGE analysis. SDS-PAGE was performed using 7.5% polyacrylamide gels on which BSA (**8**, 300 and 600ng), thiolated BSA (**10**, 500 and 1000 ng), the conjugate (**3** or **4**, 500-2500ng) and Dual Color Marker mixture (containing 37, 50, 75, 100, 150 and 250 kDa markers) were applied after boiling in sample buffer for 5 minutes. Stacking onto the running gel was performed in 30 minutes at 90V. Run time 90-120 minutes at 120V. Visualization of proteins and conjugates was achieved after staining overnight with Coomassie Brilliant Blue followed by washing three or four times with milliQ over a period of 24 hrs.



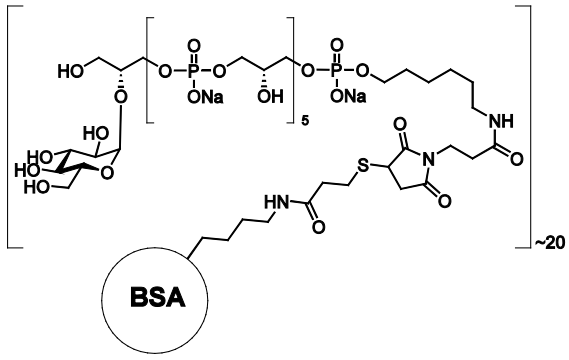
Glucosylated glycerol phosphate hexamer maleimide (6)

To a solution of TA **1** (7.0 mg, 5.3 µmol) in a mixture of saturated aqueous $NaHCO_3$ (100 µl) and H_2O (300 µl) was added *N*-succinimidyl-3-maleimido propionate **5** (28.4 mg, 107 µmol, dissolved in 800 µl 3/2 MeCN/1,4-dioxane). After stirring overnight at RT, the mixture was diluted with H_2O (~5 ml) and washed with EtOAc (20 ml). The organic layer was extracted once with 5 ml of H_2O and the combined aqueous layers concentrated under reduced pressure until ~1 ml was left. This solution was applied to a size exclusion column (Sephadex HW40, eluent: 0.05 M Et_3NHOAc) after which the fractions corresponding to the TA fragment were evaporated and, subsequently, lyophilized three times. The crude product (in ~0.5 ml H_2O) was then eluted through a small column containing Dowex Na^+ cation-exchange resin (type: 50WX4-200, stored on 0.5 M NaOH in H_2O, flushed with H_2O and MeOH before use). After concentration *in vacuo* the purified product was coevaporated three times with 1% AcOH (~5 ml) in order to regain the maleimide in the non-hydrolyzed form. Finally, after lyophilization TA maleimide **6** (5.3 mg, 3.6 µmol, 67%) was obtained as an amorphous off-white solid. ^{31}P NMR (161.7 MHz, D_2O, hydrolyzed maleimide): δ = 1.2 - 1.3 (5P), 1.4 (1P); ^{1}H NMR (600 MHz, D_2O, hydrolyzed maleimide): δ = 1.23 - 1.33 (m, 4H, 2 x CH_2 hexylspacer), 1.39 - 1.45 (m, 2H, CH_2 hexylspacer), 1.52 - 1.58 (m, 2H, CH_2 hexylspacer), 2.38 (t, 2H, *J* = 6.6 Hz, CH_2 maleimidopropionyl), 3.09 (t, 2H, *J* = 6.9 Hz, CH_2-N hexylspacer), 3.33 (at, 1H, *J* = 9.5 Hz, H-4), 3.39 (t, 2H, *J* = 6.6 Hz, CH_2-N maleimidopropionyl), 3.46 (dd, 1H, *J* = 3.9 Hz, 9.9 Hz, H-2), 3.65 - 4.00 (m, 36H, H-3, H-5, H-6, H-6', CH_2-O hexylspacer, 6 x CH glycerol, 12 x CH_2 glycerol), 5.10 (d, 1H, *J* = 3.8 Hz, H-1), 5.83 (d, 1H, *J* = 12.2 Hz, CH maleimide), 6.24 (d, 1H, *J* = 12.3 Hz, CH maleimide); HRMS: $C_{37}H_{72}N_2O_{39}P_6 + H^+$ requires 1355.2211, found 1355.2225.



Kojibiosylated glycerol phosphate hexamer maleimide (7)

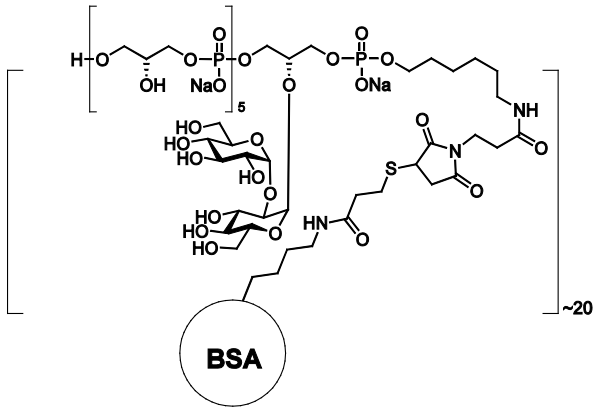
To a solution of kojibiosyl TA **2** (10.0 mg, 6.42 μmol) in a mixture of saturated aqueous $NaHCO_3$ (300 μl) and H_2O (300 μl) was added *N*-succinimidyl-3-maleimido propionate **5** (23.3 mg, 87.4 μmol, dissolved in 1.0 ml 3/2 MeCN/1,4-dioxane). After stirring overnight at RT, the mixture was diluted with H_2O (~5 ml) and washed with EtOAc (20 ml). The organic layer was extracted once with 5 ml of H_2O and the combined aqueous layers concentrated under reduced pressure until ~1 ml was left. This solution was applied to a size exclusion column (Sephadex HW40, eluent: 0.05 M Et_3NHOAc) after which the fractions corresponding to the TA fragment were evaporated and, subsequently, lyophilized three times. The crude product (in ~0.5 ml H_2O) was then eluted through a small column containing Dowex Na^+ cation-exchange resin (type: 50WX4-200, stored on 0.5 M NaOH in H_2O, flushed with H_2O and MeOH before use). After concentration *in vacuo* the purified product was coevaporated three times with 1% AcOH (~5 ml) in order to regain the maleimide in the non-hydrolyzed form. Finally, after lyophilization TA maleimide **7** (9.11 mg, 5.52 μmol, 86%) was obtained as an amorphous off-white solid. ^{31}P NMR (161.7 MHz, D_2O, hydrolyzed maleimide): δ = 0.9 (1P), 1.1 (1P), 1.2 - 1.3 (3P), 1.3 (1P); ^1H NMR (600 MHz, D_2O, hydrolyzed maleimide): δ = 1.25 - 1.33 (m, 4H, 2 x CH_2 hexylspacer), 1.41 - 1.47 (m, 2H, CH_2 hexylspacer), 1.54 - 1.59 (m, 2H, CH_2 hexylspacer), 2.39 (t, 2H, *J* = 6.6 Hz, CH_2 maleimidopropionyl), 3.10 (t, 2H, *J* = 6.9 Hz, CH_2-N hexylspacer), 3.34 - 3.42 (m, 4H, 2 x H-4, CH_2-N maleimidopropionyl), 3.49 - 3.56 (m, 2H, CH*H* glycerol, H-2), 3.59 - 3.63 (m, 2H, CH*H* glycerol, H-2), 3.67 - 4.01 (m, 37H, 5 x CH glycerol, 11 x CH_2 glycerol, CH_2-O hexylspacer, 2 x H-3, 2 x H-5, 4 x H-6), 4.10 - 4.15 (m, 1H, CH glycerol), 5.10 (d, 1H, *J* = 3.7 Hz, H-1), 5.39 (d, 1H, *J* = 3.5 Hz, H-1), 5.84 (d, 1H, *J* = 12.3 Hz, CH maleimide), 6.25 (d, 1H, *J* = 12.3 Hz, CH maleimide); HRMS (hydrolyzed form): $C_{43}H_{84}N_2O_{45}P_6$ + Na^+ requires 1557.2664, found 1557.2677.



Glucosyl TA-BSA conjugate(3)

To a solution of freshly prepared[18] thiolated BSA (**10**, 68 nmol protein, max 2.9 μmol thiol, ~4.7 mg) in phosphate buffer saline (PBS, pH 7.2, 1.65 ml) was added TA **6** (5.2 mg, 3.5 μmol, in 400 μl PBS). After stirring for 20 hrs at RT, 3-maleimidopropionic acid (0.65 mg, 3.8 μmol, in 150 μl PBS) was added. After overnight reaction the solution was transferred to a Slide-A-Lyzer dialysis cassette (20 kDa MW cut off, capacity 3 ml) and dialyzed overnight versus PBS (3l) and milliQ (2 x 3l, overnight). The resulting solution was lyophilized giving the target glucosylated hexaglycerol phosphate-BSA conjugate **3** (~8 mg) as an amorphous white solid. The protein:sugar ratio was found ~1.5:1 and was determined by UV_{280} and a phenol/H_2SO_4 assay as described above. SDS-PAGE analysis was performed as described in the general procedures and shows a broad band around 95 kDa (see Figure 2, gel A, lane 8,9). These combined results indicate the presence of ~20 TA fragments per molecule of BSA. Details SDS-

PAGE (see also figure 2, gel A); lane 1: BSA (600ng), lane 2: dual color mix (37, 50, 75, 100, 150, 250 kDa markers), lane 3: BSA (300 ng) and **10** (500 ng), lane 4: **10** (500 ng), lane 5: **10** (1000 ng), lane 6: **10** (500 ng), lane 7: dual color mix, lane 8: **3** (1200 ng), lane 9: **3** (2400 ng).



Kojibiosyl TA-BSA conjugate(4)
To kojibiosyl TA **7** (3.4 mg, 2.0 μmol) was added a solution of freshly prepared[18] thiolated BSA (**10**, 46 nmol protein, max 2.0 μmol thiol, ~3.1 mg) in phosphate buffer saline (PBS, pH 7.2, 1.39 ml). After stirring for 20 hrs at RT, 3-maleimidopropionic acid (0.34 mg, 2.0 μmol, in 100 μl PBS) was added. After overnight reaction the solution was transferred to a Slide-A-Lyzer dialysis cassette (20 kDa MW cut off, capacity 3 ml) and dialyzed overnight versus PBS (3l) and milliQ (2 x 3l, overnight). The resulting solution was lyophilized giving the target kojibiosylated hexaglycerol phosphate-BSA conjugate **4** (~5 mg) as an amorphous white solid. The protein:sugar ratio was found ~1.5:1 and was determined by UV_{280} and a phenol/H_2SO_4 assay as described above. SDS-PAGE analysis was performed as described in the general procedures and shows a broad band around 100 kDa (see Figure 2, gel B, lane 5-7). These combined results indicate the presence of ~20 kojibiosyl TA fragments per molecule of BSA. Details SDS-PAGE (see also figure 2, gel B); lane 1: BSA (600ng), lane 2: BSA (300 ng) and **10** (500 ng), lane 3: **10** (600 ng), lane 4: **10** (1000 ng), lane 5: **10** (600 ng) and **4** (600 ng), lane 6: **4** (600 ng), lane 7: **4** (1200 ng), lane 8: dual color mix (37, 50, 75, 100, 150, 250 kDa markers).

Rabbit immunization[6]: Rabbits were immunized according to literature precedents using 2 injections of 100 μg s.c. of either BSA or conjugate **3** or **4**. in incomplete Freund adjuvant, followed by 9 i.v. injections over 3 weeks. All serum samples were heat-inactivated to remove complement activity. For all experiments, serum from the final bleed of the rabbit was used.

Opsonophagocytic assays[6]: These assays were performed as described by Theilacker *et al.* White blood cells (WBCs) were collected by dextran sedimentation from fresh human blood collected from healthy adult volunteers. Baby rabbit serum (Cedarlane Laboratories, Hornby, Ontario, Canada) diluted 1:15 in Roswell Park Memorial Institute medium (RPMI)–fetal bovine serum was used as the complement source. Unless stated otherwise, bacteria (*E. faecalis* 12030, *E. faecium* E1162, *S. aureus* MW2) grown overnight on tryptic soy agar were adjusted to an optical density at 650 nm (OD_{650}) of 0.1 with fresh TSB and allowed to grow to an OD_{650} of 0.4. For comparison of opsonophagocytosis of *S. aureus* strains in different growth phases, an additional culture was grown overnight to late stationary phase, and the bacterial concentration was adjusted to an OD of 0.4 before the assay. Equal volumes of bacterial suspension (2.5 x 10^7 per mL), leukocytes (2.5 x 10^7 per mL), complement source (1.7% final concentration), and immune rabbit serum (anti-BSA, anti-**3** or anti-**4**) at the dilutions indicated in figures 3-6 were combined and incubated on a rotor rack at 37 °C for 90 minutes. After incubation, live bacteria were quantified by agar culture of serial dilutions. Controls included tubes lacking serum, tubes containing complement but lacking serum and WBCs, and tubes containing serum and WBCs but lacking complement. For all bacterial strains tested in the study, no significant killing (<5%)

was observed in the presence of complement alone. The percentage of killing was calculated by determining the mean number of colony-forming units (CFUs) in tubes without WBCs to the number of CFUs in tubes with serum samples, leukocytes, complement, and bacteria.

Opsonophagocytic inhibition assays: anti-**3** serum was diluted to a concentration of 1:1000 (final concentration) giving ~90% killing in the OPA. This antiserum was incubated at 4 ^{o}C for 60 minutes with 0.8-100 μg/mL of inhibitor. After this incubation step, the OPA was continued as described above. The percentage of inhibition of killing was calculated by determining the ratio of the CFUs surviving in the tubes with inhibitor to the CFUs surviving in the tubes without inhibitor.

Rat endocarditis experiments: The experiments were performed following a procedure described by Lee *et al.*[20] using either normal rabbit serum (control) or anti-**3** serum.

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