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Synthesis and immunochemical characterization of protein conjugates of carbohydrate and carbohydrate-mimetic peptides as experimental vaccines

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Abstract—The peptides DRPVPY and MDWNMHAA, which were identified as mimics of the cell-surface polysaccharides of *Streptococcus* Group A and *Shigella flexneri* Y, respectively, were used in this study to develop experimental vaccines directed against these two bacteria. Both oligopeptides were synthesized employing the Fmoc solid-phase strategy and linked via the amino end to a bifunctional linker, diethylsquarate. These adducts were then conjugated to the two carrier proteins, bovine serum albumin (BSA) and tetanus toxoid (TT) to yield the peptide conjugate vaccines. The average level of incorporation of DRPVPY and MDWNMHAA on TT was 65% and 75%, respectively, whereas that of both peptide haptens on BSA was 100%. A polysaccharide conjugate against *S. flexneri* Y, which comprises about 10 tetrasaccharide repeating units, was also prepared based on reductive amination at the reducing end with 1,3-diaminopropane, followed by coupling of the aminated polysaccharide to diethylsquarate, and subsequent coupling of the adduct to TT. An average incorporation of 73% of polysaccharide haptens was achieved. The glycoconjugate and the oligopeptide conjugates were shown to bind effectively to the respective monoclonal antibodies directed against the cell-surface polysaccharides.
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1. Introduction

Many strategies are being explored to manipulate the immune system to better fight a pathogen.[1] One of the most efficient and cost-effective methods is a prime/boost strategy with vaccines.[2] Several types of vaccines, which comprises either an attenuated strain of the infectious organism, killed organisms, part of the organism, or a weakened form of a toxin that it produces, have been developed and used.[2–4] However, the possibility of infection with these agents has led to their replacement by subunit vaccines, which contain only the antigens that trigger the immune system.[4,5]

Carbohydrates that coat the surfaces of many pathogenic microorganisms, such as bacteria, as well as being important in bacterial survival and virulence within the host, are also one of the main biomolecules that are recognized by the immune system.[6–9] Therefore, targeting carbohydrate antigens is a promising avenue in order to develop efficient vaccines. Some polysaccharide-based vaccines have been successfully developed, but generally show weak immunogenic effects, poor responses in infants, the elderly, and in immunodeficient persons.[6–9] Polysaccharide–protein conjugates, on the other hand, have proven effective in several cases,[9] and well-defined oligosaccharide-conjugate vaccines have also been explored with a view to eliciting discriminating immune responses.[10] However, the complex structures of polysaccharide conjugates, their production, and characterization,[11,12] make alternative approaches, to drive specific T-cell dependent immune responses against carbohydrate antigens, highly desirable.

Peptide mimics of carbohydrates have potential as surrogate ligands for traditional carbohydrate vaccines.[13] Several studies have successfully identified peptide sequences, through phage-displayed library screening, that specifically mimic certain carbohydrate structures;[13] in certain cases, these peptides could also induce an immune response against the original carbohydrate antigen.[13] Carbohydrate-mimetic peptides, which are

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easier to synthesize, also have the potential of eliciting a more targeted immune response and therefore present attractive candidates as vaccines, especially in cases involving autoimmune responses.[13]

We have targeted two bacteria to illustrate the potential of the method. The first, the Group A *Streptococcus* (GAS), causes streptococcal pharyngitis (strep throat), some forms of pneumonia, toxic shock syndrome, and necrotizing fasciitis or flesh-eating disease.[14,15] Polysaccharide-conjugate vaccines have been used in immunization of animals and the bactericidal activity of human sera (containing antibodies to GAS polysaccharide) against several strains of GAS in vitro has been demonstrated.[16] The second bacterial strain, *Shigella flexneri* Y is another virulent bacteria that causes bacillary dysentery by invading the colonic mucosa.[17] Although this particular serogroup is not a significant human pathogen, it nevertheless serves as a useful model system. The cell-wall polysaccharide of GAS is made up of a branched trisaccharide repeating unit, L-Rha-α-(1 → 2)-[D-GlcNAc-β-(1 → 3)]-α-L-Rha (Fig. 1a),[18,19] while the *O*-polysaccharide of *S. flexneri* Y is comprised of the repeating unit (→2)-α-L-Rha-α-(1 → 2)-α-L-Rha-α-(1 → 3)-α-L-Rha-α-(1 → 3)-β-D-GlcNAc-(1 →) (Fig. 1c).[20,21] Screening of a phage-displayed peptide library with the anti-carbohydrate antibody SA-3, directed against GAS polysaccharide yielded the peptide sequence DRPVPY (Fig. 1b).[22] Similarly, an octapeptide with sequence MDWNMHAA (Fig. 1d),[22] was identified as a ligand for the anti-*S. flexneri* Y antibody SYA/J6. This antibody is ideally suited for characterizing the conjugates synthesized in the present work against *S. flexneri* Y, since it has been the subject of extensive ligand mapping and crystallography studies by Bundle et al.[23,24]

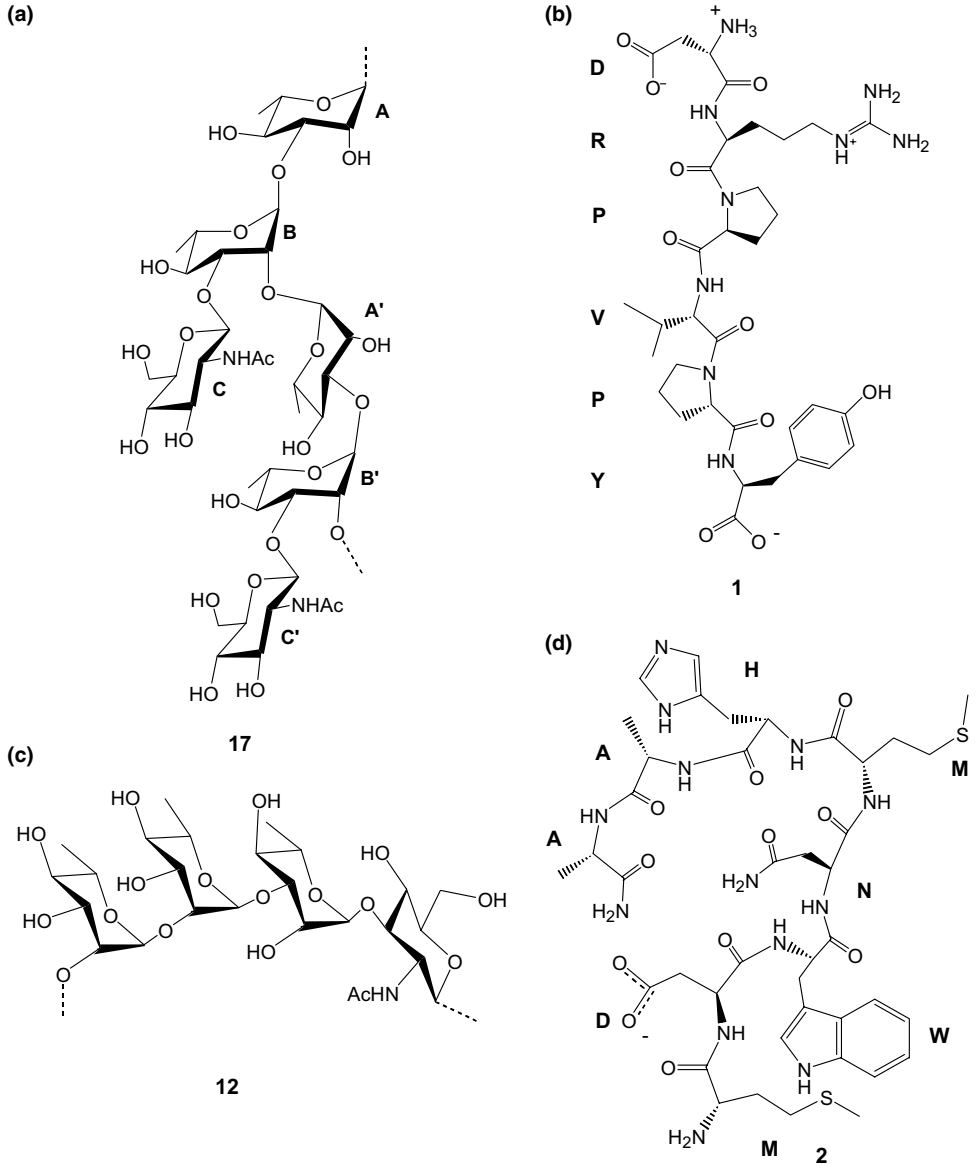


Figure 1. Structures of the (a) the cell-wall polysaccharide of Group A *Streptococcus*, (b) its peptide mimic, (c) the *O*-polysaccharide of *S. flexneri* Y, and (d) its peptide mimic.

Both oligopeptides DRPVPY and MDWNMHAA have been found to specifically bind to their corresponding antibodies used to screen peptide libraries.[22] Our long-term objective is to test the immunogenicity of the corresponding peptide-based vaccines and the cross-reactivity of immune sera with the bacterial polysaccharide. Accordingly, it was necessary to first prepare peptide conjugates and characterize them immunochemically.

The method used in the coupling of the immunizing peptide with a carrier protein is an important factor to consider when preparing peptide antigens. This factor is of concern because the flexible peptide may adopt a different preferred conformation when attached to the carrier protein.[25] The position of attachment of a peptide to the carrier protein, dictated by the conjugation method, affects the reactivity of the resulting conjugated peptide toward its complementary antibody.[26–28] Several reports exist in which peptides were conjugated via the side chain of lysine,[27] cysteine,[27,29] aspartic acid,[28] glutamic acid,[28] threonine[30] or serine.[30]

In the cases at hand, NMR and molecular modeling studies of the bound conformations of DRPVPY[31] and MDWNMHAA,[32] and replacement analysis of MDWNMHAA[33] had indicated that all the amino acids, except aspartic acid in DRPVPY and methionine (1) in MDWNMHAA, formed part of the critical epitopes. We reasoned therefore, that on conjugation of the two oligopeptides DRPVPY and MDWNMHAA to the carrier proteins, bovine serum albumin (BSA) and tetanus toxoid (TT), via the N-termini of the peptides would lead to conjugated oligopeptides that would still be recognized by the respective antibodies.

We thus embarked on a program to synthesize the carbohydrate-mimetic peptide conjugates using the two peptide sequences DRPVPY and MDWNMHAA, and describe here our findings. We chose to explore a strategy that had been very successful for the preparation of oligosaccharide–protein conjugates, namely the use of the bifunctional linker, diethylsquarate.[34–36] The application of this methodology to peptide–protein conjugates is unprecedented, to the best of our knowledge. We report in the present work the synthesis of oligopeptide–ethylsquarate adducts, linked directly via the N-termini of the peptides, followed by their conjugation to the carrier proteins, BSA, and TT. We also report the synthesis of the *S. flexneri* Y *O*-polysaccharide conjugate of TT for use in a prime/boost vaccination protocol. Finally, the immunochemical evaluation of both the glycoconjugate and the oligopeptide conjugates as ligands for their respective antibodies is described.

2. Results and discussion

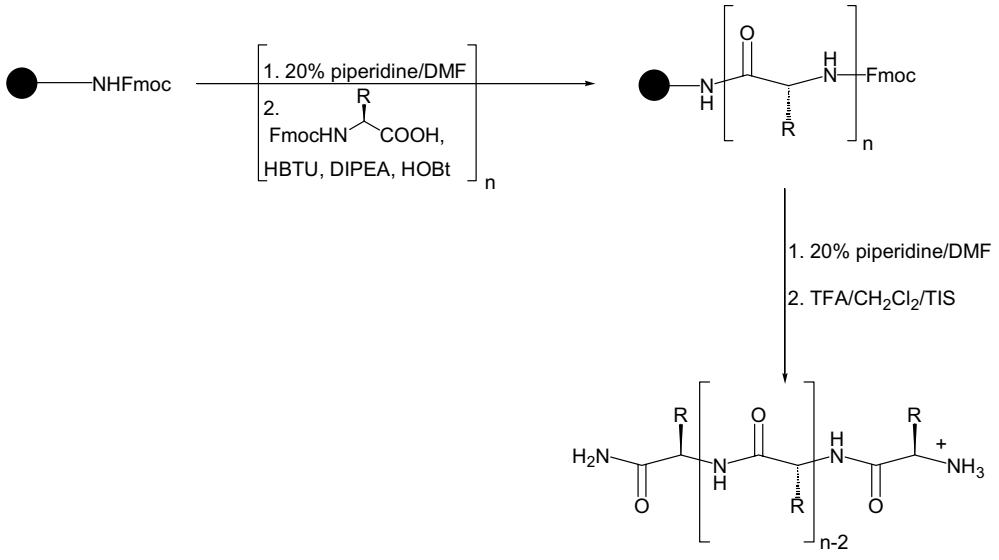
2.1. Synthesis

Since an amide functionality was desired at the C-termini of the peptides, the peptide sequences were assembled on Rink amide MBHA resin[37,38] employing Fmoc chemistry[39] and the HBTU/HOBt/DIPEA coupling strategy.[40] When the peptide chains were completed, the Fmoc protecting group was removed and cleavage/deprotection was carried out using TFA–triisopropylsilane–CH_2Cl_2, where the triisopropylsilane served as a scavenger.[37] Acid labile side chain protecting groups were utilized so that simultaneous side chain deprotection and cleavage of the peptide could be achieved. The peptides **1**–**3** were purified by HPLC on a C_{18} reverse-phase column (gradient elution CH_3CN/H_2O) and, after lyophilization, they were obtained as white powders, in yields ranging from 44% to 60% (Scheme 1).

The hexapeptide H–DRPVPY–NH_2 (**1**) and the octapeptide H–MDWNMHAA–NH_2 (**2**) were then conjugated to the two carrier proteins bovine serum albumin (BSA) and tetanus toxoid (TT), as described below (Schemes 2 and 3). The octapeptide H–MDWNPHAA–NH_2 (**3**), which was found to bind with a very low affinity to the anti-carbohydrate antibody SYA/J6 with respect to H–MDWNMHAA–NH_2 (**2**),[33] was also conjugated to BSA (Schemes 2 and 3) to provide a negative control in immunochemical experiments.

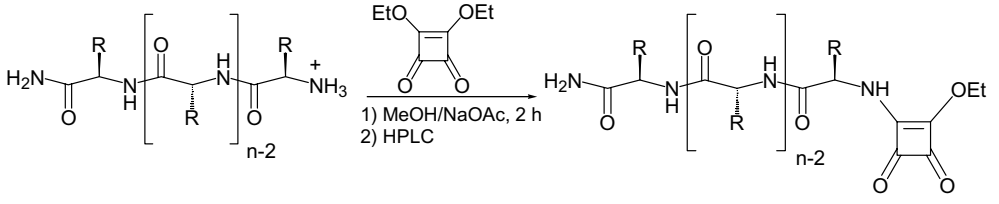
We examined the use of the bifunctional linker, 3,4-diethoxy-3-cyclobutene-1,2-dione (diethylsquarate), to link the peptides to the carrier proteins BSA and TT; this linker was found to be successful in the coupling of oligosaccharide–squarate adducts with protein carriers.[34–36] When the peptides **1**–**3** were added to diethylsquarate in methanol at room temperature, no reaction occurred, most probably because the amino termini of the peptides were protonated. Addition of small portions of sodium acetate to the reaction mixtures initiated the reactions and after 1 h, there was complete disappearance of the free peptides, as judged by MALDI-TOF mass spectrometry. After purification by HPLC and lyophilization, the peptide–squarate adducts **4**–**6** were obtained as white powders in yields ranging from 55% to 76% (Scheme 2). Both the free peptides **1**–**3** and the peptide–squarate adducts **4**–**6** were characterized by 1D and 2D (TOCSY) NMR spectroscopy and MALDI-TOF mass spectrometry. The peptide–squarate adducts **4**–**6** were then coupled to the carrier proteins BSA and TT via the conjugate addition of the ε-amino groups of the lysines of the protein to the monoethyl squarate (Scheme 3).

The first coupling reaction was attempted with the squarate–MDWNPHAA–NH_2 (**6**), and BSA, and resulted in the incorporation of an average of three peptide haptens (11%) in the conjugate **9**. The reaction was repeated and the average level of incorporation of the oligopeptide could be improved to 20 (71%) when the volume as well as the molar concentration of the carbonate buffer used in the second trial were reduced. In the coupling of squarate–DRPVPY–NH_2 (**4**) and squarate–MDWNMHAA–NH_2 (**5**) to BSA (Scheme 3), similar reaction conditions were applied but the two oligopeptide–squarate adducts **4** and **5** were added directly to the BSA, dissolved in a minimum volume of



compound	n	peptide	%
1	6	DRPVPY	60
2	8	MDWNMHAA	48
3	8	MDWNPHAA	40

Scheme 1.



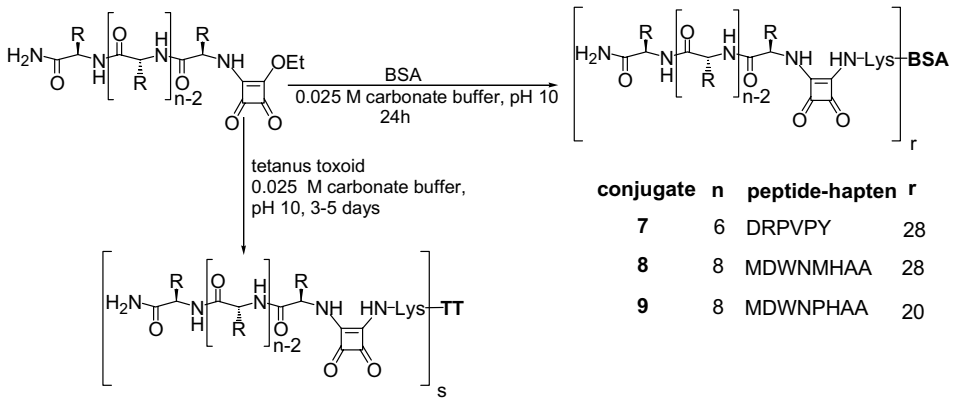
compound	n	peptide-squarate adduct	%
4	6	DRPVPY-squarate	76
5	8	MDWNMHAA-squarate	63
6	8	MDWNPHAA-squarate	55

Scheme 2.

buffer. MALDI-TOF mass spectrometry showed an average incorporation of 28 haptens per BSA molecule (100%) for both oligopeptide conjugates **7** and **8**.

The peptide–squarate adducts **4** and **5** were added to tetanus toxoid in carbonate buffer, and the reaction mixtures were stirred for 3–5 days at room temperature and monitored by MALDI-TOF mass spectrometry. When no further increase in the incorporation of the peptides on the protein was observed, the reaction mixtures were dialyzed against distilled water and lyophilized to give the tetanus toxoid–squarate–peptide conjugates **10** and **11** as white powders (Scheme 3). The DRPVPY and MDWNMHAA haptens were incorporated on the protein in yields of 65% and 75%, respectively.

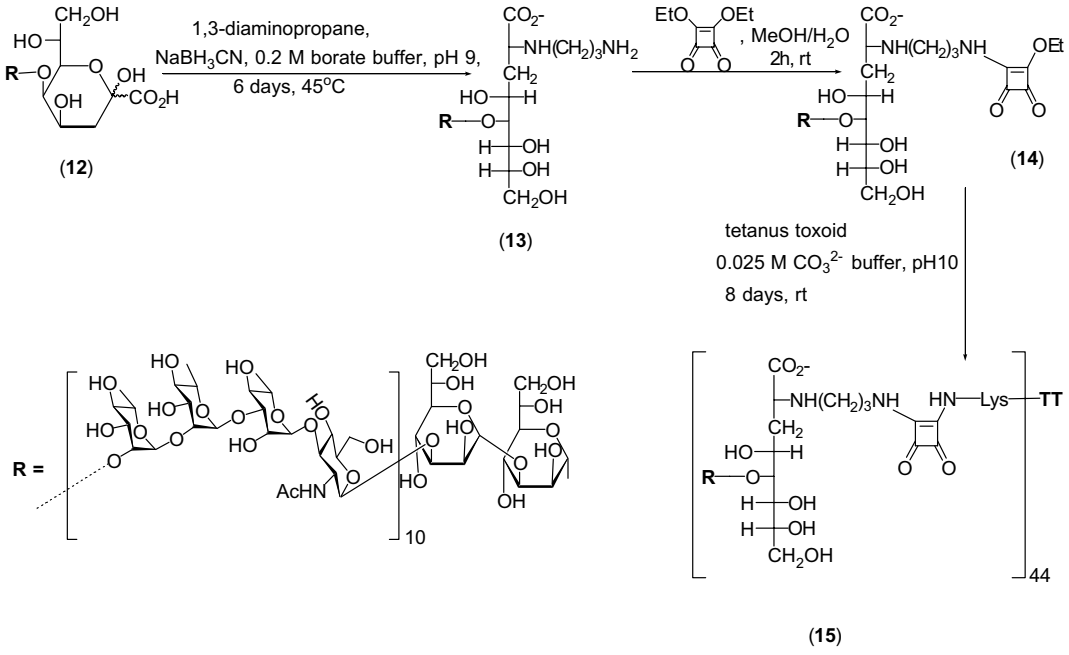
For the immunological studies, a polysaccharide-conjugate antigen of *S. flexneri* Y, which comprises about 10 oligosaccharide repeating units,[41] was prepared from *O*-polysaccharide supplied by D. R. Bundle (University of Alberta). An amino functionality was first introduced to the KDO residue of the core region of the polysaccharide **12** at the reducing end by reductive amination with 1,3-diaminopropane,[42] followed by coupling of the aminated polysaccharide **13** to diethylsquarate and, subsequent coupling of the resulting adduct **14** to the carrier protein TT (Scheme 4). The reductive amination reaction was performed for 6 days, as reported in the literature,[42] and after dialysis against water, gave a mixture of aminated polysaccharide **13** and unreacted polysaccharide **12** as a white powder (Scheme 4). The aminated product **13** could be purified on a Biogel P-2



conjugate	n	peptide-hapten	r
7	6	DRPVPY	28
8	8	MDWNMHAA	28
9	8	MDWNPHAA	20

conjugate	n	peptide-hapten	s
10	6	DRPVPY	39
11	8	MDWNMHAA	45

Scheme 3.



Scheme 4.

column eluted with 20 mM pyridylacetic acid buffer, pH 5.4,[42] but was used directly in the next step. The presence of the amino group on the aminated polysaccharide **13** was determined using the Kaiser test.[43] The reaction of the aminated polysaccharide **13** with diethylsquarate was carried out in MeOH/H_2O (2:1) at room temperature for 2 h. The reaction mixture was then dialyzed and lyophilized to give the aminated polysaccharide–squarate adduct **14**, containing some of the unreacted polysaccharide **12**, as a white powder (Scheme 4). The aminated polysaccharide–squarate adduct **14** was not isolated but was used directly in the coupling reaction with TT in carbonate buffer. The reaction mixture was stirred for 8 days at room temperature, then dialyzed against distilled water to remove the unreacted polysaccharide **12** and the squarate adduct **14**, and lyophilized to give the TT–squarate–polysaccharide adduct **15** as a white powder (Scheme 4). The sugar content of the glycoconjugate **15** was assessed by the method of Dubois et al.,[44] and the level of incorporation of the polysaccharide hapten on the protein was determined to be 73% (44 haptens).

2.2. Immunochemistry

The binding affinity of the protein conjugates **8**, **9**, **11**, and **15** was investigated by competitive ELISA, in which

these conjugates in solution were allowed to inhibit binding of the antibody SYA/J6 to *S. flexneri* Y lipopolysaccharide (LPS), as the solid phase antigen (both antibody and LPS gifts from D. R. Bundle, University of Alberta). Samples of the antibody were allowed to equilibrate with the peptide conjugates **8**, **9**, and **11**, and for comparison, with the polysaccharide **12**, the polysaccharide conjugate **15**, and octapeptide H–TDWNMHAA–NH_2[33] (**16**), which binds as well as H–MDWNMHAA–NH_2(**2**).[33] These solutions were then added to a polystyrene plate coated with *S. flexneri* Y lipopolysaccharide. Binding of the conjugates **8**, **9**, **11**, and **15** to the antibody was indicated by a reduction in the amount of antibody bound to the solid phase (measured using a secondary antibody–peroxidase conjugate). No inhibition of binding was observed in control experiments using TT or BSA at concentrations equal to or greater than those of the protein conjugates. The results of the assay are summarized in Table 1 and Figure 2.

Conjugation of the octapeptide **2** to either tetanus toxoid or BSA resulted in an increase in binding affinity of roughly 10-fold: IC_{50} values of 2.2 and 2.5 μM for **8** and **11**, respectively, in contrast to a value of 19 μM for **2**,[32] or 33.5 μM for **16**. In contrast, the protein–polysaccharide conjugate **15** and the polysaccharide **12** bound with roughly equal affinity. This may reflect the difference between a polyvalent antigen (**12**) and the monovalent

Table 1. IC_{50} values for the inhibition of binding of the monoclonal antibody SYA/J6 to *S. flexneri* Y lipopolysaccharide by peptide and polysaccharide ligands, and by their protein conjugates

Compound	IC_{50} (μM)
8	2.2
9	n.d.[a]
11	2.5
12	<0.5
15	1.1
16	33.5

[a] 50% inhibition was not attained.

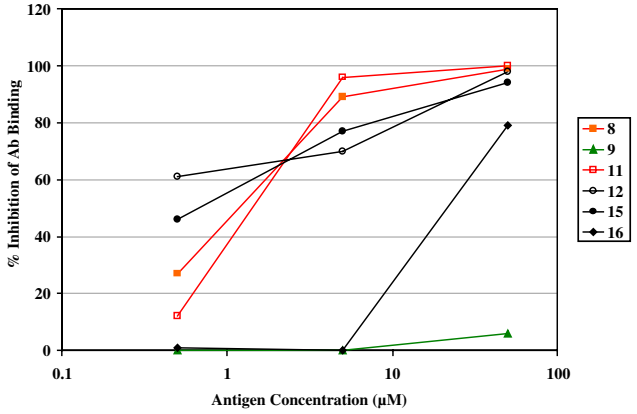


Figure 2. Inhibition of antibody binding to *S. flexneri* Y lipopolysaccharide by peptide and polysaccharide antigens, and their protein conjugates. In the case of protein conjugates, concentrations refer to the equivalent molar concentration of the free antigen.

octapeptide antigens (**2** and **16**). The 10-fold increase in binding affinity achieved by presenting the octapeptide in a multivalent fashion will likely result in greater immunogenicity of compounds, **8** and **11**, as surrogate anti-*S. flexneri* Y vaccines.

The binding affinity of protein conjugates **7** and **10** of the peptide H–DRPVPY–NH_2 (**1**), the molecular mimic of the Group A *Streptococcus* (GAS) cell-wall polysaccharide, was also investigated by competitive ELISA. However, no solution-phase inhibitors were able to reduce the binding of the anti-*Streptococcus* antibody to killed GAS bacteria on the solid phase. The inhibitors tested included the cell-wall polysaccharide **17**[18,19] (Fig. 1a), the pentasaccharide **18**,[35] corresponding to the cell-wall polysaccharide of Group A *Streptococcus* (Fig. 3a), TT–sq–pentasaccharide conjugate **19** (Fig. 3b),[45] synthesized in an analogous fashion to that described for a hexasaccharide–tetanus toxoid conjugate,[35] the peptide H–DRPVPY–NH_2 (**1**), as well as the protein conjugates **7** and **10**. The strong binding observed is likely due to high avidity of the multivalent IgM antibody for the bacteria on the solid phase. In test reactions, a small amount of inhibition (5–25%) by GAS cell-wall polysaccharide was observed when the antibody concentration was reduced to 20 or 200 pM and when the plate was coated with GAS cells at 5×10^7 or 5×10^6 cells/mL However, since a strong base-line (complete inhibition) could not be demonstrated, we decided to measure binding of the peptide conjugates **7** and **10** by direct ELISA.

Direct ELISA using plates coated with the peptide conjugates **7** and **10**, as well as the TT–sq–pentasaccharide conjugate **19** (Fig. 3b), showed that SA-3 bound strongly to **7** and **10**, with nearly equal affinity as that for **19**. The optical density values observed are reported in Table 2.

3. Conclusions

An efficient protocol has been developed to synthesize protein conjugates containing oligopeptides that can mimic the polysaccharides found on the cell surfaces of *Streptococcus* Group A and *S. flexneri* Y. A protein–polysaccharide conjugate corresponding to the *O*-polysaccharide of *S. flexneri* Y was also synthesized. Both the carbohydrate mimetic peptide conjugates and the glycoconjugate were shown to bind effectively to the respective monoclonal antibodies directed against the cell-surface polysaccharides. These conjugates will now be tested as experimental vaccines.

4. Experimental

4.1. Synthesis

4.1.1. General methods. The Fmoc amino acids used were purchased from Novabiochem and the other reagents from Aldrich Chemical Co. DMF was freed of amines by concentrating under high vacuum and was

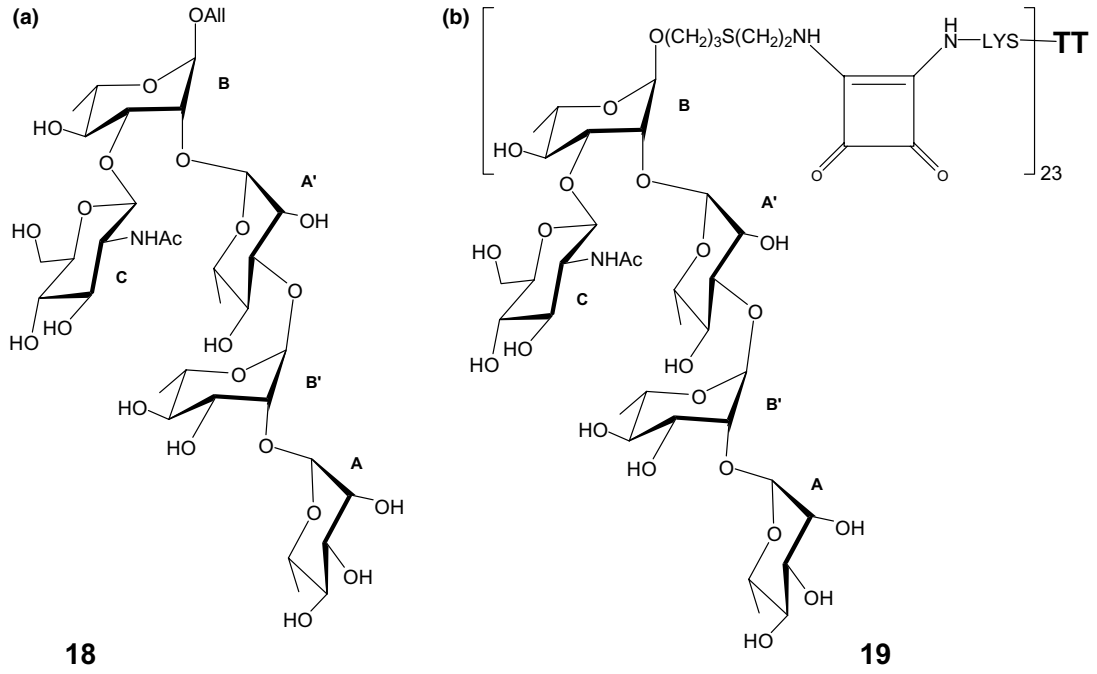


Figure 3. (a) Pentasaccharide fragment B(C)A′B′A corresponding to the cell-wall polysaccharide of Group A *Streptococcus*, (b) tetanus toxoid–squarate–pentasaccharide conjugate.

Table 2. Optical density values indicating binding of mAb SA-3 to protein conjugates **7**, **10**, and **19** in a solid phase assay

	Solid-phase protein conjugate	$\Delta OD \times 1000$[a]
7	BSA–sq–DRPVPY–NH_2	875
10	TT–sq–DRPVPY–NH_2	872
19	TT–sq–Pentasaccharide	995
—	TT	4
—	BSA	11

[a] Values are $(A_{405}-A_{490})\times 1000$, after subtraction of the background value of 7 for wells coated with skim milk solution.

then distilled and stored over molecular sieves whereas the other solvents were distilled according to standard procedures.[46] The oligopeptides sequences were synthesized on the Rink Amide [4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-MBHA resin[37,38](Novabiochem) (0.73 mmol/g substitution level), using standard 9-fluorenyl-methoxycarbonyl (Fmoc) chemistry[39] employing 2-[1-H-benzotriazole-1-yl]-1.1.13.3-tetramethyluronium hexafluorophosphate/1-hydroxybenzotriazole (HBTU/HOBt) coupling strategy.[40] The Kaiser ninhydrin[43] (5% ninhydrin in ethanol, 80% phenol in ethanol and 2% 0.001 M aq KCN in pyridine) assay for amino group was used to monitor both the Fmoc–amino acid coupling and the Fmoc deprotection reactions. 1D and 2D NMR spectra were recorded on Bruker AMX 400 and AMX 600 spectrometers, respectively, in 10% D_2O in water. Chemical shifts, which were referred to external DSS [3-(trimethylsilyl)-1-propanesulfonic acid], and coupling constants were obtained from a first-order analysis of one-dimensional spectra and ^1H assignments were based on TOCSY experiments. MALDI-TOF mass spectra were obtained for some samples dispersed in a 2,5-dihydroxybenzoic acid matrix and others in 3,5-dimethoxy-4-hydroxy cinnamic acid matrix, on a Perseptive Biosystems Voyager DE instrument. High-resolution mass spectra were LSIMS (FAB), run on a Kratos Concept H double focusing mass spectrometer at 10,000 RP.

4.1.2. Antibodies and polysaccharide antigens. The purified monoclonal antibody SYA/J6 created and characterized by the group of D. R. Bundle, together with *O*-polysaccharide and lipopolysaccharide (*S. flexneri* Y) samples were generous gifts prepared according to the methods published by this group.[23,24,42]

4.2. Synthesis of the oligopeptides 1–3

4.2.1. General procedure. Rink Amide MBHA resin (200 mg, loading = 0.73 mmol/g) was swelled in DMF (10 mL) for 2 h. A solution of piperidine (20%) in DMF (10 mL) was added to the suspension and the flask was shaken gently for 1 h using a mechanical shaker. After the suspension was filtered, the resin was washed with DMF (5 × 10 mL). A solution of Fmoc–Ala–OH (140 mg, 0.45 mmol), in the synthesis of the octapeptides **2** and **3** and Fmoc–Tyr(*t*Bu)–OH (207 mg, 0.45 mmol) in the case of H–DRPVPY–NH_2 (**1**) in DMF (10 mL) preactivated with HBTU (160 mg, 0.42 mmol) and HOBT (30.3 mg, 0.44 mmol) was then added to the suspension followed by the addition of five drops of DIPEA. The flask was shaken with a mechanical shaker. The coupling reaction was monitored by withdrawing a sample of the resin, washing it with DMF and carrying out a Kaiser test. After the reaction was completed, the suspension was filtered and the resin was washed with

DMF (5×10 mL). Successive deprotection of the Fmoc group and coupling of the appropriate Fmoc–amino acid were repeated until the peptide sequence Fmoc–MDWNMHAA–resin, Fmoc–MDWNPHAA–resin, and Fmoc–DRPVPY–resin were obtained. After removal of the Fmoc group (20% piperidine in DMF) from the N-terminal of the peptide–resin moiety, the latter was washed with DMF (5×10 mL), and dichloromethane (3×10 mL). A solution of trifluoroacetic acid, dichloromethane, and triisopropylsilane (7:7:1) was added to the peptide-resin and it was shaken with a mechanical shaker for 3 h. After the resin was filtered and washed with fresh 95% TFA (2×5 mL), the filtrate was combined and concentrated under vacuum. The peptide residues were then purified by reversed-phase HPLC (Gradient elution, acetonitrile/water) on a C_{18} column. The appropriate fractions of the synthesized peptides **1**–**3** were combined and concentrated under reduced pressure and the remaining solutions were lyophilized to afford the oligopeptides **1**–**3** as white crystals.

4.2.2. H-Asp-Arg-Pro-Val-Pro-Tyr-NH_2 (H–DRPVPY–NH_2) (1). White solid (66 mg, 60% yield). ^{1}H NMR (90% H_2O/10% D_2O) δ 8.77 (d, $J = 6.4$ Hz, Arg NH), 8.32 (d, $J = 7.6$ Hz, Val NH), 7.96 (d, $J = 7.3$ Hz, Tyr NH), 7.21 (t, $J = 5.7$ Hz, Arg side chain NH), 7.15 (2H, d, $J = 8.7$ Hz, Tyr 2,6-ArH side chain), 6.80 (2H, d, $J = 8.7$ Hz, Tyr 3,5-ArH side chain), 4.52–4.43 (3H, m, Arg α-H, Tyr α-H, Pro-3 α-H), 4.38 (1H, m, Val α-H), 4.31 (1H, m, Pro-5 α-H), 4.21 (1H, t, $J = 7.9$ Hz, Asp α-H), 3.83 (2H, m, Pro-3 δ-H), 3.65 (2H, m, Pro-5 δ-H), 3.21 (2H, dd, $J = 12.8$ and 6.56 Hz, Arg-H), 3.03 (2H, m, Tyr β-H), 2.92 (1H, dd, $J = 17.8$ and 4.7 Hz, Asp β-H), 2.78 (1H, dd, $J = 17.8$ and 8.6 Hz, Asp β-H), 2.28 (1H, m, Pro-3 β-H), 2.22 (1H, m, Pro-5 β-H), 2.10–1.93 (4H, m, Pro-3 γ-H, Val β-H, Pro-3 β-H), 1.90–1.80 (3H, m, Pro-5 γ-H, Pro-5 β-H), 1.78–1.62 (4H, m, Arg β-H, Arg γ-H), 0.96 (3H, d, $J = 7.4$ Hz, Val γ-CH_3), 0.93 (3H, d, $J = 7.6$ Hz, Val γ-CH_3); MALDI-TOF MS: (m/z) calcd for $C_{34}H_{54}N_{10}O_9$: 747.86 [M^++H]; Found: 747.48 [M^++H].

4.2.3. H-Met-Asp-Trp-Asn-Met-His-Ala-Ala-NH_2 (H–MDWNMHAA–NH_2) (2). White crystals (68 mg, 47.8% yield). ^{1}H NMR (90% H_2O/10% D_2O) δ 10.15 (br s, Trp ring NH), 8.77 (d, $J = 7.4$ Hz, Asp NH), 8.59 (s, His H-2), 8.41 (d, $J = 7.6$ Hz, His NH), 8.36 (d, $J = 6.5$ Hz, Trp NH), 8.32 (d, $J = 5.9$ Hz, Ala-8 NH), 8.25 (d, $J = 5.8$ Hz, Ala-7 NH), 8.19 (d, $J = 7.5$ Hz, Asn NH), 8.05 (d, $J = 6.8$ Hz, Met NH), 7.63 (d, $J = 8.0$ Hz, Trp H-4), 7.53 (br s, C-terminal Ala amide NH), 7.51 (br s, Asn side chain NH), 7.50 (d, $J = 8.1$ Hz, Trp H-7), 7.28 (2H, s, His H-4, Trp H-2), 7.24 (dt, $J = 7.1$ and 0.9 Hz Trp, H-6), 7.15 (dt, $J = 7.1$ and 0.8 Hz, Trp H-5), 7.05 (br s, C-terminal Ala amide NH), 6.87 (br s, Asn side chain NH), 4.28 (3H, m, Ala α-H, Ala α-H, Met α-H), 4.03 (t, $J = 6.8$ Hz, Met α-H), 3.32 (dd, $J = 14.8$ and 6.6 Hz, Trp β-H), 3.27 (dd, $J = 15.5$ and 5.9 Hz, His β-H), 3.22 (dd, $J = 14.9$ and 7.8 Hz, Trp β-H), 3.15 (dd, $J = 15.5$ and 8.8 Hz, His β-H), 2.91 (dd, $J = 17.0$ and 6.6 Hz, Asp β-H), 2.79 (dd, $J = 17.0$ and 7.3 Hz, Asp β-H), 2.67 (dd $J = 15.8$ and 6.8 Hz, Asn β-H), 2.58 (dd, $J = 15.8$ and 6.9 Hz, Asn β-H), 2.48 (ddd, $J = 13.7$, 8.1 and 5.5 Hz, Met γ-H), 2.43–2.36 (2H, m, Met γ-H and Met γ-H), 2.27 (td, $J = 13.7$ and 6.8 Hz, Met γ-H), 2.07 (3H, s, Met ε-CH_3), 2.03–1.84 (4H, m, Met β-CH_2, Met β-CH_2), 1.96 (3H, s, Met ε-CH_3), 1.41 (3H, d, $J = 7.1$ Hz, Ala-8 β-CH_3), 1.38 (3H, d, $J = 7.3$ Hz, Ala-7 β-CH_3); MALDI-TOF MS: (m/z) calcd for $C_{41}H_{59}N_{13}O_{11}S_2$: 975.13 [$M^+$+H]; Found: 975.25 [$M^+$+H]. HRMS: ($m/z$) Calcd for $C_{41}H_{59}N_{13}O_{11}S_2$: 974.3977 [$M^+$+H]; Found: 974.3967 [$M^+$+H].

4.2.4. H-Met-Asp-Trp-Asn-Pro-His-Ala-Ala-NH_2 (H–MDWNPHAA–NH_2) (3). White crystals (60 mg, 43.7% yield). ^{1}H NMR (90% H_2O/10% D_2O) δ 10.17 (br s, Trp ring NH), 8.84 (d, $J = 7.3$ Hz, Asp NH), 8.61 (d, $J = 1.4$ Hz, His H-2), 8.38 (d, $J = 6.9$ Hz, Trp NH), 8.28 (d, $J = 6.2$ Hz, Ala NH), 8.27(d, $J = 8.0$ Hz, His NH), 8.13 (d, $J = 8.1$ Hz, Asn NH), 7.99 (d, $J = 5.8$ Hz, Ala NH), 7.62 (d, $J = 7.9$ Hz, Trp H-4), 7.57 (br s, Asn side chain NH), 7.52 (br s, C-terminal Ala amide NH), 7.50 (d, $J = 8.2$ Hz, Trp H-7), 7.31 (s, His H-4), 7.25 (d, $J = 2.22$ Hz, Trp H-2), 7.25 (dt, $J = 7.1$ and 1.0 Hz Trp, H-6), 7.16 (dt, $J = 7.0$ Hz and 1.0 Hz, Trp H-5), 7.05 (br s, C-terminal Ala amide NH), 6.94 (br s, Asn side chain NH), 4.28 (dq, $J = 7.3$ and 3.0 Hz, Ala α-H), 4.27 (dq, $J = 7.2$ and 2.5 Hz, Ala α-H), 4.12 (dd, $J = 8.7$ and 3.8 Hz, Pro α-H), 4.08 (t, $J = 6.7$ Hz, Met α-H), 3.58 (2H, ddd, $J = 9.7$ Hz, 7.4 and 5.0 Hz, Pro δ-CH_2), 3.31–3.25 (3H, m, Pro δ-H, Trp β-H, His β-H), 3.21 (dd, $J = 14.5$ and 6.9 Hz, Trp β-H), 3.14 (dd, $J = 15.5$ and 9.4 Hz, His β-H), 2.90 (dd, $J = 17.0$ and 6.3 Hz, Asp β-H), 2.79 (dd, $J = 17.0$ and 7.8 Hz, Asp β-H), 2.74 (dd $J = 15.8$ and 8.4 Hz, Asn β-H), 2.55 (dd, $J = 15.8$ and 6.2 Hz, Asn β-H), 2.48 (ddd, $J = 13.7$, 7.8 and 6.7 Hz, Met γ-H), 2.39 (ddd, $J = 13.7$, 7.5 and 6.7 Hz, Met γ-H), 2.14 (br, m, Proβ-H), 2.10–2.01 (2H, m, Met β-CH_2), 2.02 (3H, s, Met ε-CH_3), 1.84 (br, m, Pro, β-H), 1.72 (2H, m, Pro γ-CH_2), 1.41 (3H, d, $J = 7.3$ Hz, Ala β-CH_3), 1.38 (3H, d, $J = 7.1$ Hz, Ala β-CH_3); MALDI-TOF MS: (m/z) calcd for $C_{41}H_{57}N_{13}O_{11}S$: 941.06 [$M^+$+H]; Found: 941.06 [$M^+$+H]. HRMS: ($m/z$) calcd for $C_{41}H_{57}N_{13}O_{11}S$: 940.4099 [$M^+$+H]; Found: 940.4108 [$M^+$+H].

4.3. Synthesis of the peptide–squarate adducts 4–6

4.3.1. *N*-(3,4-Dione-2-ethoxycyclobutene)-DRPVPY-NH_2 (sq–DRPVPY–NH_2) (4). To a solution of diethylsquarate (sq) (17 μL, 1.5 equiv) in freshly distilled MeOH (1 mL), H–DRPVPY–NH_2 (**1**) (57.4 mg, 0.077 mmol), dissolved in MeOH (2 mL), was added followed by the addition of sodium acetate (15 mg). The reaction mixture was stirred at room temperature for 1.5 h. After the reaction was completed, the solution was concentrated under reduced pressure and the residue was purified by reverse-phase HPLC (Gradient elution, acetonitrile/water). After freeze-drying of the appropriate fractions, the sq–DRPVPY–NH_2 adduct (**4**) was obtained as a white powder (51 mg, 76%). ^{1}H NMR (90% H_2O/10% D_2O) δ

8.93 (d, $J = 7.8$ Hz, Asp NH), 8.35 (d, $J = 6.9$ Hz, Arg NH), 8.16 (d, $J = 7.6$ Hz, Val NH), 7.95 (d, $J = 7.5$ Hz, Tyr NH), 7.28 (d, $J = 7.2$ Hz, Arg side chain NH), 7.10 (2H, d, $J = 8.7$ Hz, Tyr 2,6-ArH side chain), 6.79 (2H, d, $J = 8.7$ Hz, Tyr 3,5-ArH side chain), 5.34–4.52 (4H, m, Arg α-H, Asp α-H, Tyr α-H, Pro-3 α-H), 4.38 (1H, m, Val α-H), 4.29 (1H, m, Pro-5 α-H), 3.75 (2H, m, Pro-3 δ-H), 3.68–3.50 (4H, m, CH_2-squarate, Pro-5 δ-H), 3.15 (2H, dd, $J = 12.6$ and 6.7 Hz, Arg δ-H), 2.95 (2H, m, Tyr β-H), 2.85 (2H, dd, $J = 17.5$ and 8.3 Hz, Asp β-H), 2.25–2.10 (2H, m, Pro-3 β-H, Pro-5 β-H), 2.09–1.85 (4H, m, Pro-3 -H, Val β-H, Pro-3 β-H), 1.87–1.72 (3H, m, Pro-5 γ-H, Pro-5 β-H), 1.70–1.50 (4H, m, Arg -H, Arg γ-H), 1.13 (3H, t, $J = 7.1$ Hz, CH_3–squarate), 0.89 (6H, d, $J = 7.4$ Hz, Val γ-CH_3); MALDI-TOF MS: (m/z) calcd for $C_{40}H_{58}N_{10}O_{12}$: 871.96 [M^++H]; Found: 871.73 [M^++H].

4.3.2. *N*-(3,4-Dione-2-ethoxycyclobutene)-MDWNMHAA-NH_2 (sq–MDWNMHAA–NH_2) (5) and *N*-(3,4-dione-2-etoxycyclobutene)-MDWNPHAA-NH_2 (sq–MDWNPHAA–NH_2) (6). The octapeptides H–MDWNMHAA–NH_2 (**2**) (3 mg, 9.2 μmol) and H–MDWNPHAA–NH_2 (**3**) (13 mg, 13.8 μmol) were treated with diethylsquarate (sq) as described above. After purification by HPLC, the peptides–squarate adducts **5** (2.2 mg, 63%) and **6** (8.2 mg, 55%) were obtained as white powders. ^{1}H NMR for *N*-(3,4-dione-2-ethoxycyclobutene)-MDWNMHAA-NH_2 (**5**) (90% H_2O/10% D_2O) δ 10.10 (br s, Trp ring NH), 8.61 (s, His H-2), 8.50 (d, $J = 7.6$ Hz, Met-1 NH), 8.38 (d, $J = 7.3$ Hz, Asp NH), 8.32 (d, $J = 7.7$ Hz, His NH), 8.25 (d, $J = 6.5$ Hz, Trp NH), 8.21 (d, $J = 5.6$ Hz, Ala-8 NH), 8.18 (d, $J = 5.7$ Hz, Ala-7 NH), 8.15 (d, $J = 7.5$ Hz, Asn NH), 8.02 (d, $J = 6.8$ Hz, Met NH), 7.63 (d, $J = 7.9$ Hz, Trp H-4), 7.54 (d, s, C-terminal Ala amide NH), 7.53 (br s, Asn side chain NH), 7.51 (d, $J = 8.1$ Hz, Trp H-7), 7.31 (s, His H-4), 7.29 (s, Trp H-2), 7.27 (dt, $J = 7.1$ and 0.9 Hz Trp, H-6), 7.18 (dt, $J = 7.1$ and 0.8 Hz, Trp H-5), 7.06 (br s, C-terminal Ala amide NH), 6.86 (br s, Asn side chain NH), 4.30 (3H, m, Ala α-H, Ala α-H, Met α-H), 3.69–3.60 (m, CH_2–squarate, Met α-H), 3.38 (dd, $J = 14.6$ and 6.8 Hz, Trp β-H), 3.29 (dd, $J = 15.7$ and 5.9 Hz, His β-H), 3.20 (dd, $J = 14.7$ and 7.8 Hz, Trp β-H), 2.60–2.40 (m, His β-H, Asp β-H, Asp β-H, Asn β-H, Met-5 γ-H, Met-1 γ-H), 2.10–2.05 (6H, s, Met-5 ε-CH_3, Met-5 ε-CH_3), 2.04–1.90 (4H, m, Met-1 β-CH_2 and Met-5 β-CH_2), 1.96 (3H, s, Met-1 ε-CH_3, Met-5 ε-CH_3), 1.44 (3H, d, $J = 6.9$ Hz, Ala-8 β-CH_3), 1.40 (3H, d, $J = 7.1$ Hz, Ala-7 β-CH_3), 1.21 (3H, t, $J = 7.0$ Hz, CH_3-squarate); MALDI-TOF MS: (m/z) calcd for $C_{47}H_{63}N_{13}O_{14}S_2$: 1099.21 [$M^+$+H]; Found: 1099.35 [$M^+$+H].

^{1}H NMR for *N*-(3,4-dione-2-ethoxycyclobutene)-MDWNPHAA-NH_2 (**6**) (90% H_2O/10% D_2O) δ 10.10 (br s, Trp ring NH), 8.59 (d, $J = 7.5$ Hz, Asp NH), 8.55 (d, $J = 7.8$ Hz, Met-1 NH), 8.53 (d, $J = 1.4$ Hz, His H-2), 8.32 (d, $J = 7.2$ Hz, Trp NH), 8.28 (d, $J = 5.8$ Hz, Ala-8 NH), 8.19 (d, $J = 7.8$ Hz, His NH), 8.11 (d, $J = 7.9$ Hz, Asn NH), 7.96 (d, $J = 5.8$ Hz, Ala-7 NH), 7.55 (d, $J = 7.33$ Hz, Trp H-4), 7.48 (br s, C-terminal Ala amide NH), 7.44 (d, $J = 8.1$ Hz, Trp H-7), 7.25–7.16 (3H, m, Trp H-2, His H-4, Trp, H-6), 7.10 (dt, $J = 7.2$ Hz and 1.1 Hz, Trp H-5), 7.01 (br s, C-terminal Ala amide NH), 6.81 (br s, Asn side chain NH), 5.05 (dq, $J = 7.2$ and 3.1 Hz, Ala-8 α-H), 4.85 (dq, $J = 7.2$ and 2.3 Hz, Ala-7 α-H), 4.25 (dd, $J = 8.5$ and 3.9 Hz, Pro α-H), 4.00 (t, $J = 6.7$ Hz, Met α-H), 3.55–3.45 (3H, m, Pro δ-H, CH_2–squarate), 3.31–3.25 (3H, m, Pro δ-H, Trp β-H, His β-H,), 3.15–3.11 (2H, m, Trp β-H, His β-H), 2.83 (dd, $J = 16.8$ and 6.5 Hz, Asp β-H), 2.70 (dd, $J = 17.0$ and 7.5 Hz, Asp β-H), 2.60 (dd, $J = 15.8$ and 8.4 Hz, Asn β-H), 2.53 (dd, $J = 15.8$ and 6.2 Hz, Asn β-H), 2.43 (ddd, $J = 13.5$, 7.4 and 6.6 Hz, Met γ-H), 2.35 (ddd, $J = 13.6$, 7.5 and 6.6 Hz, Met γ-H), 2.22–2.15 (br, m, Proβ-H, Met β-H), 2.01 (3H, s, Met ε-H), 1.94 (br, m, Pro, β-H), 1.84 (2H, m, Pro γ-H), 1.35 (3H, d, $J = 7.2$ Hz, Ala-8 β-CH_3), 1.31 (3H, d, $J = 7.2$ Hz, Ala-7 β-H), 1.10 (3H, t, $J = 7.2$, CH_3-squarate); MALDI-TOF MS: (m/z) calcd for $C_{47}H_{62}N_{13}O_{14}S$: 1066.15 [$M^+$+H]; Found: 1066.31 [$M^+$+H].

4.4. Preparation of the BSA–sq–peptide conjugates 7–9

4.4.1. Synthesis of the BSA–sq–MDWNPHAA–NH_2 conjugate (9). To a solution of BSA (4.13 mg, 0.0626 μmol) in 0.1 M carbonate buffer pH 10 (0.3 mL) was added sq–MDWNPHAA–NH_2 (**6**) (3 mg, 2.82 μmol) dissolved in the same buffer (0.2 mL). The reaction mixture was stirred at room temperature and the incorporation of the peptide–squarate adduct **6** was monitored by MALDI-TOF mass spectrometry. After 5 days, when no further increase of the peptide haptens was observed, the reaction mixture was dialyzed against distilled water using an Amicon ultrafiltration cell equipped with a Diaflo membrane (NMWL: 3000). The residue dissolved in water was lyophilized to give the BSA–sq–peptide conjugate **9** as a white powder (4.30 mg). The peptide conjugate **9** was shown to contain 3 octapeptide haptens per BSA molecule (11%).

4.5. Increasing the number of sq–MDWNPHAA–NH_2 (6) haptens on BSA

4.5.1. Synthesis of the BSA–sq–MDWNPHAA–NH_2 conjugate (9). Sq–MDWNPHAA–NH_2 (**6**) (1.45 mg, 1.36 μmol) in 0.025 M carbonate buffer pH 10 (3 drops) was added to a solution of BSA (2 mg, 0.0303 μmol) in 0.025 M carbonate buffer (0.15 mL). The reaction mixture was stirred at room temperature and after 5 days, the level of the incorporation of the peptide haptens was 71%. The BSA–sq–MDWNPHAA–NH_2 conjugate **(9)** was obtained as white powder after dialysis and lyophilization.

4.5.2. Synthesis of the BSA–sq–DRPVPY–NH_2 (7) and BSA–sq–MDWNMHAA–NH_2 (8) conjugates. The two sq–peptide adducts **4** and **5** were conjugated to BSA following the same procedure as described above. The amount of buffer solution used to dissolve the BSA was reduced and the sq–peptide adducts **4** and **5** were added directly to the BSA. The peptide contents of both conjugates **7** and **8** after 24 h stirring were 28 (100%

incorporation of the peptides), based on the results obtained from MALDI-TOF mass spectrometry. Both peptide conjugates **7** and **8** were obtained as white powders.

4.6. Preparation of the tetanus toxoid–sq–peptide (TT–sq–peptide) conjugates 10 and 11

4.6.1. Synthesis of TT–sq–DRPVPY–NH_2 (10). Tetanus toxoid (2 mL, 3 mg/mL), available in tris buffer, was dialyzed against 0.025 M carbonate buffer pH 10 (3 × 5 mL) and concentrated to a minimum volume of 0.3–0.5 mL. The sq–DRPVPY–NH_2 (**4**) (3 mg, 3.4 mmol) was then added and the reaction mixture was stirred for 3 days at room temperature. The peptide conjugate **10** was then dialyzed against distilled water and after lyophilization, it was obtained as a white powder (1.28 mg). MALDI-TOF mass spectrometry showed an incorporation of 39 peptide haptens (65%).

4.6.2. Synthesis of TT–sq–MDWNMHAA–NH_2 (11). TT–sq–MDWNMHAA–NH_2 (**11**) was prepared as described above for the preparation of the TT–sq–DRPVPY–NH_2 (**10**). The peptide conjugate **11** was obtained as a white powder and its peptide content was 45 hapten molecules per TT (75%).

4.7. Preparation of the polysaccharide glycoconjugate 15

4.7.1. Synthesis of the aminated polysaccharide 13. The *S. flexneri* Y *O*-polysaccharide (**12**) was polydisperse with 7–15 repeating units, as assessed by SDS-gel chromatography;[41] we chose an average value of 10 repeating units for our calculations. To the *O*-polysaccharide **12** (2 mg, 0.267 μmol), dissolved in 0.2 M sodium borate buffer, pH 9, (0.2 mL) was added dropwise a solution of 1,3-diaminopropane dihydrochloride (3.92 mg, 0.027 mmol), in 0.1 mL of the same buffer containing sodium cyanoborohydride (0.257 mg, 3.33 μmol). The reaction mixture was stirred for 6 days at 45 °C. It was then diluted with water and dialyzed to give a mixture of aminated polysaccharide **13** and unreacted polysaccharide **12** as a white powder. The product was assayed for the presence free amino group[43] and sugar.[44] The aminated polysaccharide **13** was not separated from the unreacted polysaccharide **12** but was used directly in the next reaction.

4.7.2. Synthesis of the aminated polysaccharide–squarate adduct 14. The mixture of the unreacted polysaccharide **12** and aminated polysaccharide **13** was treated with diethylsquarate (sq) (51 μL, 0.297 μmol) in MeOH/H_2O (2:1) for 2 h at room temperature. It was then dialyzed against distilled water (5 × 5 mL) and the residue dissolved in water was lyophilized to give a mixture of the squarate-aminated polysaccharide **14** and the unreacted polysaccharide **12** as a white powder. The mixture was used directly in the next coupling reaction.

4.7.3. Synthesis of the TT–sq–aminated polysaccharide 15. Tetanus toxoid (0.13 mL, 3 mg/mL) in tris buffer was dialyzed against 0.025 M carbonate buffer, pH 10, (3 × 5 mL), concentrated to 0.3 mL and then added to the mixture above. After 8 days stirring at room temperature, the reaction mixture was dialyzed against distilled water (5 × 5 mL) and the residue dissolved in water was lyophilized to give the glycoconjugate **15** as a white powder (1 mg). The polysaccharide content of the conjugate **15** was determined using the method of Dubois et al.[44] and was shown to contain 44 polysaccharide haptens (73%).

4.7.4. Dialyzes of the protein conjugates. The dialyzes of the protein–peptide conjugates **7**–**11**, the aminated polysaccharide **13**, the aminated polysaccharide squarate adduct **14**, and TT were carried out using an Amicon ultrafiltration cell equipped with a Diaflo membrane (NMWL: 3000); for the dialysis of the polysaccharide conjugate **15**, a Diaflo membrane (NMWL: 10,000) was used.

4.8. Typical analysis of oligosaccharide content

TT–sq–polysaccharide conjugate **15** (1 mg) was dissolved in distilled water (5 mL). To three test tubes, 100, 200, and 300 μL of the conjugate solution were added, respectively. The solutions in each test tube were adjusted with distilled water to 1000 μL. Phenol (25 μL, 80%) was then pipetted into the first test tube followed by rapid addition of concentrated sulfuric acid (2.5 mL). This process was repeated with the other two test tubes. The tubes were allowed to stand for 10 min, before they were shaken and placed in a water bath at 30 °C. The absorbances of the pink color was measured at 490 nm. A blank was also prepared by substituting distilled water for the sugar solution. The amount of sugar was determined by reference to a standard curve prepared using L-rhamnose and *N*-acetylglucosamine.

4.9. ELISA binding assays

4.9.1. Competitive ELISA binding assay for *S. flexneri* Y antibody. A 96-well polystyrene plate (Fisher) was coated with *S. flexneri* Y LPS (40 μL/well; 1 μg/mL in Tris–buffered saline solution (TBS; 50 mM Tris, 150 mM NaCl, pH 7.5)) by incubation overnight at 4 °C with gentle rocking. After washing four times with TBS/0.1% Tween 20, wells were blocked with skim milk solution (200 μL/well; 5% skim milk powder in TBS) for 30 min at 37 °C with gentle shaking, then washed another four times before addition of pre-mixed antibody/conjugate solutions (40 μL/well). The mAb SYA/J6 (60 nM) and conjugates **8**, **9**, **11**, and **15** (at concentrations equivalent to 0.5–50 μM of the free ligand), in skim milk solution, were allowed to pre-equilibrate by incubation overnight at 4 °C with gentle rocking. The given concentrations are the final concentrations in the assay, and therefore, solutions of the antibody and conjugates **8**, **9**, **11**, and **15** were prepared at twice the final concentration and equal

volumes (20 μL) were mixed. These solutions were added to the LPS-coated plate, which was then incubated again overnight at 4 °C with gentle rocking. The wells were washed six times with TBS/0.1% Tween 20. The secondary antibody–horseradish peroxidase conjugate (Pierce, ImmunoPure goat anti-mouse IgG (H+L), mixed with glycerol for long-term storage, and diluted 1:500 in skim milk solution; 40 μL/well) was added and the plate, which was incubated for 30 min at 37 °C with gentle shaking. The plate was washed six times with TBS/0.1% Tween 20. The concentration of bound antibody was measured by a colorimetric assay for horseradish peroxidase, as follows. To the washed plate was added a freshly prepared solution of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (0.51 mM) (ABTS; Sigma) in citric acid–phosphate buffer (61.4 mM citric acid, 77.2 mM disodium hydrogen phosphate, pH 4) containing 0.03% H_2O_2. The color was allowed to develop and was measured as the difference between absorbance at 405 and 490 nm ($A_{405} - A_{490}$); the values measured at 20 min, corresponding to maximum intensity, were used.

4.9.2. Competitive ELISA binding assay for Group A *Streptococcus* antibody. The above procedure was followed for the Group A *Streptococcus* (GAS) carbohydrate–mimetic peptide conjugates **7** and **10**, the cell-wall polysaccharide **17**, the pentasaccharide **18**, corresponding to the cell-surface of Group A *Streptococcus*, with slight modifications as follows. The plate was coated with heat-killed, pepsin-treated GAS cells (40 μL/well, overnight at 4 °C with gentle rocking); 5×10^7 cells/mL in 50 mM Na_2CO_3, pH ~ 10) to provide a solid-phase antigen. The mAb SA-3 was used at 20 nM concentration. Pre-equilibration of the antibody/ligand solutions was carried out for 2 h at room temperature, followed by 20 min at 37 °C with gentle shaking. The secondary antibody–horseradish peroxidase conjugate (Pierce, goat anti-mouse IgM (μ) was used at 1:1000 dilution in skim milk solution. Unfortunately, no inhibition of binding was observed under these conditions, likely due to high avidity of the multivalent IgM antibody for the solid phase.

In test reactions, a small amount of inhibition (5–25%) by the GAS cell-wall polysaccharide **17** was observed when the antibody concentration was reduced to 20 or 200 pM and when the plate was coated with GAS cells at 5×10^7 or 5×10^6 cells/mL. However, since complete inhibition could not be demonstrated, we decided to measure binding of the peptide conjugates **7** and **10** by direct ELISA.

4.9.3. Direct ELISA binding assay. A 96-well polystyrene plate (Fisher) was coated with protein solutions (40 μL/well; ~1 μg/well; 0.1–0.2 nM in TBS) by incubation overnight at 4 °C with gentle rocking. The plate was washed with TBS/0.1% Tween 20, blocked with skim milk solution (200 μL/well), and washed another four times with TBS/0.1% Tween 20, as described above. The antibody SA-3 (20 μL, 40 nM in skim milk solution) was added to each well, followed by another 20 μL of skim milk solution with or without GAS cells, 5×107 cells/mL, for a final antibody concentration of 20 nM. The plate was incubated for 1 h at 37 °C with gentle shaking, washed six times with TBS/0.1% Tween 20. The secondary antibody–horseradish peroxidase conjugate (goat anti-mouse IgM (μ)) was allowed to bind (20 min, 37 °C, with gentle shaking). After washing the plate six times with TBS/0.1% Tween 20, freshly prepared ABTS solution (ABTS concentration 0.36 mM) was added and the color was allowed to develop. The maximum intensity was reached at roughly 20 min and the data recorded at this time point were reported.

The concentrations of the protein conjugates **7** and **10** for coating the plate were chosen as follows. The concentration of the TT–sq–DRPVPY–NH_2 conjugate **10** was set at 1 μg/well (1 μg/40 μL), or 137 nM. The concentrations of the other conjugate **7**, were adjusted based on the molar ratios of incorporation, to provide an equal number of conjugated ligands on the solid phase; therefore, the concentration of the TT–sq–pentasaccharide conjugate **19** (Fig. 3b), was adjusted by a factor of $39/23\times137$ nM, to 232 nM; the concentration of the BSA–sq–DRPVPY–NH_2 conjugate **7** was adjusted by a factor of $39/28\times137$, or 191 nM. The negative controls TT and BSA were used at 232 and 191 nM, respectively.

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