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Multimeric bivalent immunogens from recombinant tetanus toxin H_C fragment, synthetic hexasaccharides, and a glycopeptide adjuvant

Aileen F. G. Bongat · Rina Saksena · Roberto Adamo · Yukari Fujimoto · Zenyu Shiokawa · Dwight C. Peterson · Koichi Fukase · Willie F. Vann · Pavol Kováč

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Abstract Using recombinant tetanus toxin H_C fragment (rTT-H_C) as carrier, we prepared multimeric bivalent immunogens featuring the synthetic hexasaccharide fragment of O-PS of *Vibrio cholerae* O:1, serotype Ogawa, in combination with either the synthetic hexasaccharide fragment of O-PS of *Vibrio cholerae* O:1, serotype Inaba, or a synthetic disaccharide tetrapeptide peptidoglycan fragment as adjuvant. The conjugation reaction was effected by squaric acid chemistry and monitored in virtually real time by SELDI-TOF MS. In this way, we could prepare well-defined immunogens with predictable carbohydrate–carrier ratio, whose molecular mass and the amount of *each saccharide* attached could be independently determined. The ability to prepare such neoglycoconjugates opens unprecedented possibilities for preparation of conjugate vaccines for bacterial diseases from synthetic carbohydrates.

A. F. G. Bongat · R. Adamo · P. Kováč (✉)
NIDDK, LBC, National Institutes of Health,
Bethesda, MD 20892-0815, USA
e-mail: kpn@helix.nih.gov

Y. Fujimoto · Z. Shiokawa · K. Fukase
Department of Chemistry, Graduate School of Science,
Osaka University,
Toyonaka, Osaka 560-0043, Japan

R. Saksena · D. C. Peterson · W. F. Vann
OVRR, CBER, FDA, Laboratory of Bacterial Toxins,
8800 Rockville Pike,
Bethesda, MD 20892, USA

Present Address:
R. Adamo
Novartis Vaccines and Diagnostics, s.r.l,
Siena, Italy

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Abbreviations

TT	Tetanus toxoid
rTT-H_C	Recombinant tetanus toxin fragment C
H_C	Tetanus toxin fragment C
O-PS	O-specific Polysaccharides
SELDI-TOF-MS	Surface-enhanced laser desorption time-of-flight mass spectrometry
BSA	Bovine serum albumin
NIS	N-Iodosuccinimide
PG	Peptidoglycan

Introduction

In the developing world where sanitation practices are inadequate, 131,943 cases of and 2,272 deaths from cholera were reported to the World Health Organization (WHO) in 2005. Indeed, true figures are likely to be much higher as more and more countries report cholera outbreaks each year. While WHO has advocated the use of new-generation cholera vaccines to prevent and control cholera since 1999, current vaccines for cholera are still severely limited in their application and protective capacity.

Over the years, advantages of conjugate vaccines have been widely recognized [1–3]. Many such vaccines, standalone or polyvalent, are in clinical use [2] but the first clinically useful *polyvalent* conjugate vaccine from a *synthetic* oligosaccharide and a *recombinant* protein carrier is yet to be formulated. Here we describe the *first attempt* towards that goal by preparing two *bivalent* glycoconjugate

immunogens, each containing two different antigens, that feature recombinant tetanus toxin C fragment [4] (rTT-H_C) as a carrier.

Tetanus toxoid (TT) is one of the most commonly used carrier proteins for the preparation of protein-polysaccharide conjugate vaccines. Due to its immunogenicity and protective capacity, TT has been in use since 1930's as a vaccine against tetanus. However, despite the immunogenicity associated with tetanus toxoid conjugates, there are several disadvantages to using a chemical toxoid as a carrier protein. Tetanus toxin is a ~159 kDa protein which consists of three functional domains: the catalytic N-terminal L chain, the translocational H_N chain, and the receptor-binding H_C chain [5–7], each having a molecular mass ~50 kDa. Treatment of tetanus toxin with formaldehyde, to obtain the toxoid, results in structural changes that have not been characterized. Consequently, TT does not have a well-defined structure, making it difficult to prepare and reproduce a well-defined polysaccharide conjugate. Consequently, structures and potency of the vaccines prepared in this way vary from batch to batch.

Tetanus toxin fragment C (H_C), has been shown to induce protective antibodies in animals when given by a variety of delivery systems and routes, *e.g.* parenterally [8], orally using an attenuated Salmonella delivery system [9], as a plant vaccine [10], or as DNA [11] vaccine. Consequently, H_C has been proposed as a possible replacement for the existing tetanus toxoid vaccine [12]. These advances have prompted us to attempt developing a conjugate vaccine for cholera using recombinant tetanus toxin fragment C (rTT-H_C), which can be expected to have clear advantages over the toxoid in terms of reproducibility, characterization, homogeneity and quality control of the final vaccine. The carboxyl terminal fragment of tetanus toxin (rTT-H_C) that we used herein has a molecular weight of 52,108 Da based on its amino acid sequence.

Results and discussion

We have been involved in preparation of immunogens from carbohydrates that mimic antigens of bacterial pathogens for many years. Extensive studies [13–17] on conjugation of synthetic carbohydrates to proteins by squaric acid chemistry [18] led us to conclude that this method is superior to those developed earlier. Our revised protocol for conjugation [17] works efficiently with only a small excess of the precious synthetic carbohydrate (~20%), which is comparable to that normally used in syntheses of small organic molecules.

Previously, we have found that surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF MS) can be used to monitor the progress of conjugation [15]. This brought preparation of glycoconjugates to a new level of sophistication, as it provides near real time information about the growing molecular mass of the neoglycoconjugate being formed. Consequently, individual or series [19] of tailor-made neoglycoconjugates with predetermined carbohydrate ratio can be prepared. Indeed, we have successfully prepared a large number of such constructs using the above methods and techniques [20–24]. Extrapolating on the above, we should be able to use these techniques to prepare conjugates having more than one synthetic carbohydrate chemically attached to a protein carrier, and determine the average molar content of each of the antigens attached per mole of the carrier.

The O-specific polysaccharides (O-PSs) of *Vibrio cholerae* O1, serotype Ogawa and Inaba, consist of a chain of (1→2)-linked moieties of 4-amino-4,6-dideoxy-α-D-mannopyranose (perosamine), the amino groups of which are acylated with 3-deoxy-L-*glycero*-tetronic acid. The O-PSs of the two strains differ in that the terminal, upstream perosamine moiety in the Ogawa strain carries a methoxy group at C-2 (see inset, Fig. 1). We have previously tested [25] the protective capacity of antisera produced following immunization of neonatal mice with glycoconjugates prepared from bovine serum albumin (BSA) and the terminal hexasaccharide of *Vibrio cholerae* O:1, serotype Ogawa, which had carbohydrate–protein ratios ~5:1, ~10:1, ~15:1. All three conjugates conferred protection but the 5:1 carbohydrate-BSA conjugate was most efficient.

A similar construct prepared from the Inaba hexasaccharide [20] showed immunogenicity but did not induce protection [26]. The mono and multivalent constructs described here (Fig. 1, A–D) were prepared from the same hexasaccharides whose conjugates with BSA were studied previously [15, 20]. This time, however, the nontoxic, recombinant tetanus toxin fragment C (rTT-H_C) was employed as carrier. Immunization studies involving these conjugates are in progress and should reveal differences in immunogenicity resulting from the use of a different carrier. The two multivalent conjugates (Fig. 1, C and D) both contain the Ogawa hexasaccharide (Fig. 1, inset) and, in addition, **C** contains an equimolar amount of the corresponding Inaba antigen (Fig. 1, inset), while **D** includes a chemically attached disaccharide tetrapeptidoglycan immunomodulator.

The functionalized Inaba hexasaccharide carbohydrate antigen **8**, which is part of conjugates **B** and **C** (Fig. 1) was newly synthesized as follows (Scheme 1). Reaction under thermodynamic control of thioglycoside **1** with tetrasaccharide **2** gave the fully protected hexasaccharide **3** [27]. Deprotection of **3** (→**4**→**5**→**6**) gave methyl ester **6**, which was identical (TLC, NMR) to the previously synthesized substance [20, 28]. Compound **6** was converted to the squaric acid derivative **8** conventionally [17], and the Ogawa

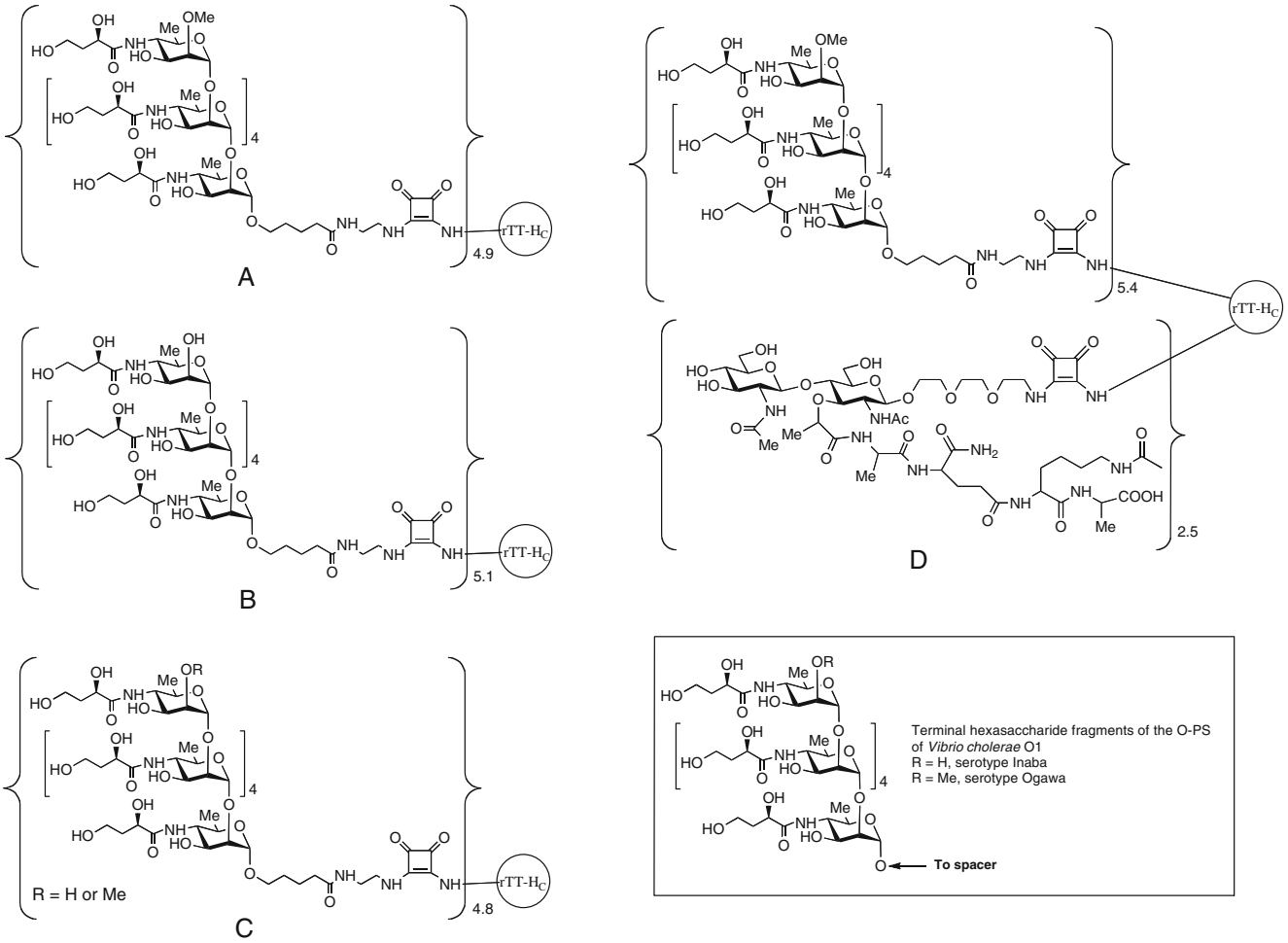
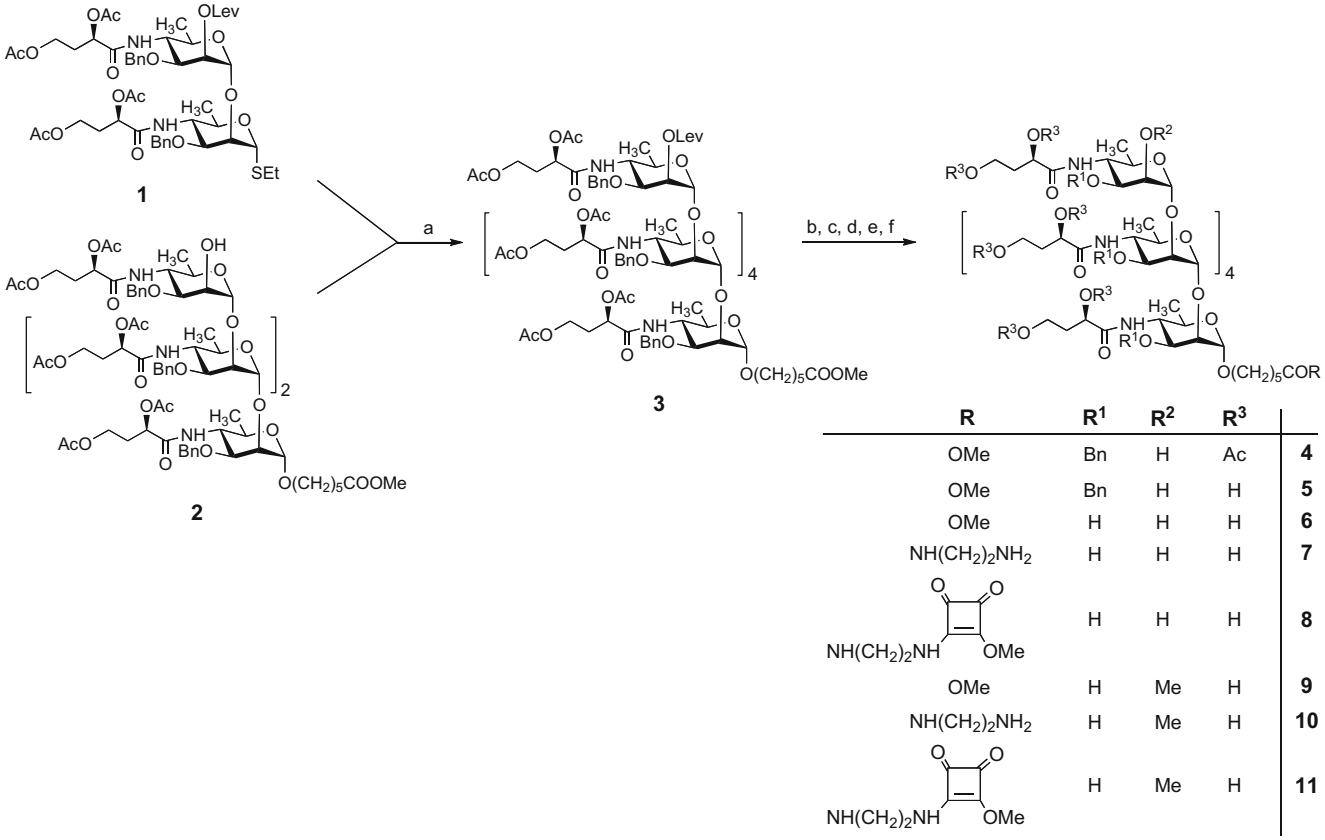


Fig. 1 Multimeric Glycoconjugates from Recombinant Tetanus Toxin C Fragment (rTT-H_C). **A** Ogawa hexasaccharide conjugate, loading 4.9; **B** Inaba hexasaccharide conjugate, loading 5.1; **C** Inaba/Ogawa hexasaccharide bivalent conjugate, combined loading 4.8; **D** Ogawa hexasaccharide/tetrapeptide glycan conjugate

hexasaccharide **9** [27] was converted through **10** [24] to **11** in a similar way.

It was suggested [29], based on results with a number of glycoconjugates, that constructs such as **A** and **B** are weak antigens and require adjuvants in order to elicit a strong protective immune response. This prompted us to examine the possibility of preparing a glycoconjugate having both an antigen characteristic of a disease-causing pathogen *and* an immunostimulant chemically attached to the same carrier protein. The product of our endeavor, conjugate **D**, is such a construct as it has the terminal determinant of the O-PS of *Vibrio cholerae* O:1, serotype Ogawa and a synthetic [30, 31] peptidoglycan (PG) fragment attached to a common carrier. Peptidoglycans and fragments thereof are known for their immunomodulating properties [31, 32]. Intercellular receptors (Nod1 and Nod2), which activate the immune system, were recently found as receptors of PG [33]. Extracted or synthesized PG fragments, such as muramyl dipeptide, have also been used as immune adjuvants [34, 35].

The peptidoglycan disaccharide tetrapeptide fragment **12** (Scheme 2), β-GlcNAc-(1→4)-β-MurNAc tetrapeptide (L-Ala-γ-D-Gln-L-Lys-D-Ala), equipped with an ethylene glycol type linker, was prepared to serve as a chemically attached adjuvant in the synthetic immunogen **D** (Fig. 1). The linker has a terminal amino group, making it amenable for conjugation by squaric acid chemistry [18, 36]. The parent structure of fragment **12** was prepared as previously described [30, 31]. Its further functionalization to contain the linker will be reported elsewhere. Briefly, an appropriately protected disaccharide (β-GlcNTroc-(1→4)-MurNTroc-1-O-α,β-trichloroacetoimidate was used to glycosylate the ethylene glycol type linker, which had a hydroxyl group at one end and a protected amino group at the other end. After introduction of the linker to the disaccharide, the tetrapeptide moiety was attached, and the resulting substance was globally deprotected to give the desired compound **12**. The latter was converted to the squaric acid monomethyl ester **13** to make it amenable to conjugation (Scheme 2).



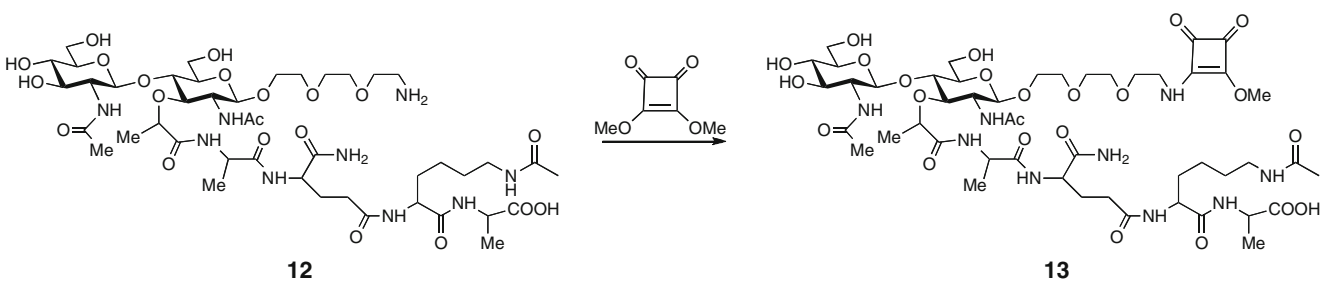
R	R^1	R^2	R^3	
OMe	Bn	H	Ac	**4**
OMe	Bn	H	H	**5**
OMe	H	H	H	**6**
$NH(CH_2)_2NH_2$	H	H	H	**7**
	H	H	H	**8**
OMe	H	Me	H	**9**
$NH(CH_2)_2NH_2$	H	Me	H	**10**
	H	Me	H	**11**

Scheme 1 **a** NIS, AgOTf, toluene, reflux; **b** H_2NNH_2•AcOH, CH_2Cl_2/MeOH, room temperature; **c** NaOMe/MeOH; **d** H_2, Pd/C, MeOH; **e** $H_2N(CH_2)_2NH_2$, 60°C; **f** dimethyl squarate, pH 7.0

The conjugation of the functionalized haptens **8** and **11** to the recombinant tetanus toxin C fragment (rTT-H_C) was done following the previously-described protocol (see Experimental for details) [17]. Conjugations leading to monovalent conjugates **A** and **B**, which were targeted to contain ~5 mol of hapten per mol of rTT-H_C carrier, were uneventful. The bivalent conjugate **C** was also targeted to have combined carbohydrate–protein ratio ~5:1. Since the structure and molecular mass of haptens **8** and **11** are very similar, **C** was prepared by conjugating an equimolar mixture of **8** and **11** to rTT-H_C. Finally, conjugate **D** was designed to contain 3 moles of **13** per carrier. However, while the carbohydrate-carrier ratio of **D** was close to what we desired, we unexpectedly had to use a large excess of hapten (total of 6 moles of **13** per carrier) to achieve a final loading of 2.5:1 (**13**:rTT-H_C). It is possible that the squarate derivative **13** was considerably less reactive than the analogous hexasaccharides **8** or **11**, or that the lysine residues that remained unchanged after attachment of the Ogawa hexasaccharide were less accessible.

Conclusion

Multivalent, relatively low molecular weight constructs have been described [37–43] but, to our knowledge,



Scheme 2 Conversion of the linker equipped, synthetic bacterial cell-wal peptidoglycan disaccharide tetrapeptide fragment **12** to the corresponding methyl squarate derivative **13**

preparation of tailored neoglycoconjugates having two different synthetic carbohydrates attached to one protein carrier has not been reported. This makes conjugate **D** principally novel in that one of the chemically attached ligands is a synthetic, disease specific antigen and the other is a synthetic molecule specifically chosen to enhance the overall immunogenicity of the resulting construct.

We have shown here that sequential conjugation of synthetic carbohydrate antigens to protein carriers can be done efficiently, and that multivalent immunogens with predetermined carbohydrate–protein ratios can be easily prepared. The neoglycoconjugates obtained are well-defined constructs, whose molecular mass and the amount of *each saccharide* attached can be independently determined. This opens unprecedented possibilities for preparation of similar multivalent constructs, thus advancing the field of developing conjugate vaccines for bacterial diseases from synthetic carbohydrates.

Experimental section

General methods All reactions were monitored by thin-layer chromatography (TLC) on Silica gel 60 coated glass slides. Column chromatography was performed by elution from columns of silica gel with the CombiFlash Companion Chromatograph (Isco, Inc.). Solvent mixtures less polar than those used for TLC were used at the onset of separation. Nuclear Magnetic Resonance (NMR) spectra were measured at 600 MHz (^{1}H) and 150 MHz (^{13}C) with a Bruker Avance 600 spectrometer. Assignments of NMR signals were made by homonuclear and heteronuclear 2-dimensional correlation spectroscopy, run with the software supplied with the spectrometers. Assignment of ^{13}C NMR spectra of some higher oligosaccharides was aided by comparison with spectra of related substances reported previously from this laboratory or elsewhere [44]. When the latter approach was used, to aid in the ^{13}C NMR signal-nuclei assignments, advantage was taken of variations of line intensity expected for oligosaccharides belonging to the same homologous series [45, 46]. Thus, spectra showed close similarity of chemical shifts of equivalent carbon atoms of the internal residues, and an increase in the relative intensity of these signals with the increasing number of D-perosamine residues in the molecule. When reporting assignment of NMR signals, nuclei associated with the 4-amido side-chain are denoted with a prime (′) and those with the spacer (linker) are denoted with a double prime (″). When reporting assignments of NMR signals, sugar residues in oligosaccharides are serially numbered, beginning with the one bearing the aglycone, and are identified by a Roman numeral superscript (*e.g.* H-5^{VI}) in listings of signal assignments. Nuclei assignments without any Roman numeral superscript (*e.g.* H-5) denote nuclei that may belong to any sugar residue. NMR spectra of amines **7** and **9** as well as those of squaric acid derivatives **8** and **11** were very similar to those of the corresponding esters **6** and **9**, respectively. Spectra of amines showed that, due to hindered rotation around the CN bond in amides [47] each of these substances was a mixture of two isomers, whose ratio varied with the structure. The presence of isomers manifested itself by splitting of signals not only of some nuclei in the linker but also signals of some ring protons and, mainly, carbons of some sugar moieties. Due to the double bond nature of the vinylogous amide group, the NMR spectra of squaric acid esters **8** and **11** showed further splitting of some signals [48]. When reporting NMR data of these substances only resonances originating from the major isomer are listed. ES-TOF MS spectra were recorder in positive mode using a Waters LCT Premier Micromass Instrument. Squaric acid dimethyl ester was purchased from Aldrich Chemical Company and recrystallized from MeOH. Solid-phase (SP) extraction tubes (Strata X) were purchased from Phenomenex. Conjugation of carbohydrates to rTT-H_C was monitored by the BioRad Protein Chip SELDI system using MP-20 chip arrays. Liquid Chromatography–Electron Spray-Ionization Mass Spectrometry (ESI-MS) was performed with a Hewlett-Packard 1100 MSD spectrometer. Gas chromatography Electron Impact Mass Spectrometry (GC-EI-MS) was performed with a Hewlett-Packard 5898A spectrometer. Solutions in organic solvents were dried with anhydrous Na_2SO_4, and concentrated at 40˚C/2 kPa.

Construction of pWVTetH$_C$ The expression plasmid pWVTetH$_C$ was constructed by amplification of the receptor-binding domain of tetanus toxin gene using the forward primer TetH$_C$SAP1 fwd (GGTGGTTGCTCTTCC AACATGGGAT- CCTCAAAAAATCTGGATTGTTGG GTT) and the reverse primer, TetH$_C$Pst1 rev (GGTGGTCTG CAGTTCATTAATCATTTGTCCATCCTTCATC). The H_C fragment gene was amplified from DNA isolated from the *Clostridium tetani* Massachusetts C2 strain as template. The gel purified PCR fragment was digested with Sap1 and Pst1 and ligated into the Sap1 and Pst1 sites of the intein expression vector pTYB11 (New England Biolabs). This yields a protein, after cleavage of the intein, with the sequence MGS at the amino terminus of TetH$_C$ fragment [49]. The resulting insert was sequenced to confirm the absence of errors due to PCR.

Purification of tetanus H_C fragment Recombinant tetanus H_C fragment has been purified as a His tagged chimer [4, 50, 51]. In this paper we have constructed expression vector encoding an amino terminal chimer of tetanus H_C fragment intein and chitin binding domain. The H_C product is

purified in high yield by cleavage of the protein and retains only 1 additional amino acid belonging to the tag.

E. coli BL21 (DE3) Star was transformed with pWVTetH$_C$ on LB ampicillin plates. The transformants were resuspended in 5 ml LB ampicillin (100 μg/ml) and the suspension used to inoculate 1.5 liter of LB ampicillin (100 μg/ml). The culture was shaken at 25°C, 120–170 rpm until the absorbance at 600 nm reached 0.6. The culture was induced by adjusting concentration of IPTG solution to 1 mM solution and shaking the solution overnight at 25°C. The cells were harvested, resuspended in 100 ml column buffer (20 mM HEPES, 500 mM NaCl pH7.8) containing 2 Complete EDTA-free protease inhibitor tablets (Roche). The cells were lysed in a French Pressure cell and centrifuged for 1 h at 27,000 g and 4°C. Nucleic acids in the lysate supernatant were digested with 2 mg DNAse and 1.5 mg RNAase for 1 h at 2–8°C.

Chitin column The lysate supernatant was loaded onto a chitin column equilibrated in column buffer. The column was washed until all unbound protein detected by Abs 280 has been eluted. To cleave the intein tag, slightly less than 1 column volume of 50 mM DTT in column buffer was introduced, and the column was capped and the mixture was stored at 16°C for 24–40 h. The protein was then eluted with column buffer and precipitated from solution by adjusting to 90% saturated ammonium sulfate.

Gel filtration The ammonium sulfate precipitate was dissolved in 1–2 ml of 20 mM ammonium acetate and further purified on a Sephacryl S-200 column (280 ml bed volume). The major peak fractions were pooled and lyophilized. The lyophilized protein was stored dry at −20°C.

5-(Methoxycarbonyl)pentyl 4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl-(1→2)-*tetrakis*[4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl]-4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranoside (6). Molecular sieves (4 Å, 0.5 g) were added under Ar (balloon) to a stirred solution of the thioglycosyl donor **1** (1.3 eq., 391 mg, 0.39 mmol) and the glycosyl acceptor **2** (0.3 mM, 536 mg) in 7:1 Toluene: DCM (ca ~15 ml) [27]. The balloon was replaced with a reflux condenser (equipped with a drying tube) and the mixture was brought to reflux. When all of the DCM could be assumed to have escaped (~15–20 min), a solid mixture of NIS (0.6 mM, 135 mg) and AgOTf (0.3 mM, 77 mg) was quickly added and the stirring was continued at the reflux temperature until TLC showed that the reaction was complete (15–20 min). After cooling to room temperature (RT), the mixture was filtered through a Celite pad into a separating funnel and partitioned between DCM and aq. sodium thiosulphate/sodium bicarbonate. The organic phase was dried, concentrated, and chromatography (1:1 hexane–acetone) gave **3** (475 mg, 69%). There was a broad singlet present in the ^{1}H NMR spectrum ($CDCl_3$) at δ 5.41, characteristic of the Lev*O*-2^{VI}, and the signals for C-6^{I-VI} in the ^{13}C NMR spectrum were at δ 18.50, 17.99 (2 C), 17.96, 17.89, and 17.77. ESI-MS calculated for $C_{138}H_{182}N_6NaO_{53}$ $[M+Na]^+$: 2794.1628, found: 2794.1631.

Hydrazine acetate (0.21 mM. 19.34 mg) in MeOH (2 ml) was added to a mixture of 2-*O*-levulinoylated compound **3** (0.2 mM, 475 mg) in DCM (18 ml), and the mixture was stirred overnight at RT, when TLC showed that the reaction was complete. The mixture was concentrated and the residue was chromatographed (2:1 CH_2Cl_2–Acetone) to give 5-(methoxycarbonyl)pentyl 3-*O*-benzyl-4-(2,4-di-*O*-acetyl-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-2-*O*-levulinoyl-α-D-mannopyranosyl-(1→2)-*tetrakis*[3-*O*-benzyl-4-(2,4-di-*O*-acetyl-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl]-3-*O*-benzyl-4-(2,4-di-*O*-acetyl-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranoside (**4**, 330 mg, 72%). The broad singlet present in the ^{1}HNMR ($CDCl_3$) spectrum of **3** at δ 5.41, characteristic of the Lev-*O*-2^{VI}, was absent. Signals for anomeric protons appeared at δ 5.10 (d, $J_{1,2}$ 2.2 Hz), 5.01 (d, $J_{1,2}$ 2.1 Hz,), 5.00 (bs), 4.97 (bs), 4.86 (bs), 4.69 (d, $J_{1,2}$ 1.8 Hz).

The foregoing alcohol **4** (330 mg, 0.11 mM) was dissolved in MeOH (~50 ml), methanolic 1 M NaOMe was added until the solution was strongly basic, and the mixture was stirred overnight at RT. After neutralization with Amberlite IR 120 (H^+) resin and conventional work-up chromatography (5:1 CH_2Cl_2–MeOH), 5-(methoxycarbonyl) pentyl 3-*O*-benzyl-4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl-(1→2)-*tetrakis*[3-*O*-benzyl-4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl]-3-*O*-benzyl-4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranoside (**5**, 325 mg, 98%) was obtained. ^{1}H NMR (CD_3OD) spectrum showed signals for anomeric protons at δ 5.11 5.09, 5.08, 5.07, 4.9, 4.78 (6 d, $J_{1,2}$~1.7 Hz). The partially overlapped six doublets for H-6^{I-VI} appeared at ~1.19 (6 H), 1.09–1.06 (12 H). ^{13}C NMR (CD_3OD): δ 103.00, 102.19, 102.09, 102.06, 102.00, 100.23 (C-1^{I-VI}), 18.63, 18.61, 18.54, 18.51, 18.42, 18.34 (C-6^{I-VI}). ESI-MS calculated for $C_{109}H_{153}N_6O_{39}$ $[M+H]^+$: 2170.0173, found: 2170.0220.

A mixture of compound **5** (320 mg) and 5% palladium on charcoal catalyst (240 mg) in MeOH (20 mL) was stirred at RT under H_2 atmosphere overnight, when TLC (1:1 CH_2Cl_2:MeOH) showed complete conversion of the starting material to a more polar product. After filtration through a Celite pad and concentration of the filtrate, chromatography gave the fully deprotected, title ester **6** (228 mg, 95%). Confidently assigned signals in the ^{1}H NMR spectrum (D_2O) were at: δ 5.17, 5.16, 5.14 (d, 1 H,

$J_{1,2}$ 1.4 Hz; bs, 2 H; 5.14, d, $J_{1,2}$ 1.3 Hz; H-$1^{II–V}$), 5.04 (d, 1 H, $J_{1,2}$ 1.8 Hz, H-1^{VI}), 4.88 (d, 1 H, $J_{1,2}$ 1.6 Hz, H-1^{I}), 4.29 (m, 6 H, H-2″), 4.10 (dd, 1 H, $J_{2,3}$ 3.2 Hz, H-2^{VI}), 4.06, 4.03 (2 dd, partially overlapped, H-3^{I},3^{VI}), 3.94–3.86 (m, 13 H, H-2^{I}, H-$4^{I–VI}$,5 $^{I–VI}$), 3.74–3.69 (m, 13 H, H-2′, H-1″a), 3.68 (s, 3 H, OCH_3), 3.53 (m, 1 H, H-1″b), 2.40 (t, 2 H, *J* 7.3 Hz, H-5″), 2.03, 1.84 (2 m, 6 H, each, H-$3'^{I–IV}$a,b), 1.62 (m, 4 H, H-2″a,b,4″a,b), 1.38 (m, 2 H, H-3″a,b), 1.17 (m, 18 H, H-$6^{I–VI}$). Confidently assigned signals in the ^{13}C NMR spectrum (D_2O) were at: δ 103.62 (C-1^{VI}), 103.62, 103.53, 103.50 (2 C, C-$1^{II–V}$), 101.19 (C-1^{I}), 80.51, 80.07, 80.02, 79.99, 79.89 (C-$2^{I–V}$), 71.90 (C-2^{VI}), 71.76 (C-$2'^{I–VI}$), 70.04–70.19 (m, 13 C, C-$3^{I–VI}$,5 $^{I–VI}$, incl. C-1″ at 70.68), 60.63 (C-$4'^{I–VI}$), 54.91 (OCH_3), 38.77 (3 C), 38.75 (2 C), 38.73 (C-3′), 36.38 (C-5″), 30.84, 26.77 (C-2″,4″), 27.64 (C-3″), 19.68, 19.63 (3 C), 19.61, 19.60 (C-$6^{I–VI}$). ESI-MS calculated for $C_{67}H_{117}N_6O_{39}$ $[M+H]^+$: 1629.7356, found: 1629.7368.

(2-Aminoethylamido)carbonylpentyl 4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl-(1→2)-*tetrakis*[4- (3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl]-4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranoside (7). A stirred solution of ester **6** (200 mg) in ethylenediamine (2 ml) was heated at 60°C overnight, after which TLC (5:0.1 MeOH–25% aq. Ammonia, or reverse phase 3:1 MeOH–water) showed complete conversion of the starting material. The mixture was concentrated and the residue was chromatographed (reverse phase, water→75% MeOH) to give the amino amide **7** (162.65 mg, 81%). Due to presence of isomeric structures (see above), the ^{1}H NMR spectrum (D_2O) showed characteristic splitting of some signals, and the resonances characteristic of the presence of the two methylene protons in the NH-CH_2-CH_2-NH_2 structural segment (more abundant isomer) were at δ 3.23 and 2.88; The corresponding ^{13}C NMR signal appeared at δ 42.33. ESI-MS calculated for $C_{68}H_{121}N_8O_{38}$ $[M+H]^+$: 1657.7782, found: 1657.7783.

1-((2-Aminoethylamido)carbonylpentyl 4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl-(1→2)-*tetrakis*-[(4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl]-(1→2)-4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranoside)-2-methoxycyclobutene-3,4-dione (8). A solution of amine **7** (120 mg, 0.072 mmol), 3,4-dimethoxy-3-cyclobutene-1,2-dione (0.11 mM, 15.4 mg) and borate buffer (0.5 M pH7.00) was stirred at RT for 1.5 h, when TLC (1:1 EtOAc–MeOH) showed complete conversion of the starting material into a UV positive, charring product. The mixture was directly chromatographed on a Strata SPE tube (10 g, H_2O→50% MeOH), to give squarate derivative **8** (106 mg, 83%). The ^{1}H NMR (D_2O) spectrum was very similar to that of the corresponding ester **6**, and contained signals for the squaric acid methyl ester group (two isomers) at δ 4.37 and 4.33. In the ^{13}C NMR (D_2O) spectrum, the corresponding, structurally significant signals were at δ 63.73 and 63.71. ESI-TOF-MS calculated for $C_{73}H_{123}N_8O_{41}$ $[M+H]^+$: 1767.7786, found: 1767.7794.

1-((2-Aminoethylamido)carbonylpentyl 4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-2-*O*-methyl-α-D-mannopyranosyl-(1→2)-*tetrakis*-[(4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl]-(1→2)-4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranoside)-2-methoxycyclobutene-3,4-dione (11). Compound **9** was treated with ethylenediamine as described [24], to give the aminoamide product **10**. Compound **10** was then converted to the squaric acid derivative **11** as described above for the **7**→**8** conversion. Definitive signals in the ^{1}H NMR spectrum (D_2O) were at: δ 5.28 (δ, 1 H, $J_{1,2}$ 1.7 Hz, H-1^{VI}), 5.27 (d, 1 H, $J_{1,2}$ 1.7 Hz, H-1), 5.25 (bt, 2 H, 2 H-1), 5.23 (bd, 1 H, $J_{1,2}$ ~1 Hz, H-1), 4.96 (bs, 1 H, H-1^{I}), 4.46 (s, OCH_3, squarate), 4.40–4.35 (m, 6 H, H-2′), 4.27–4.22 (m, H-$2^{II–V}$,3^{I}), 4.15 (dd, partially overlapped, $J_{2,3}$ 3.4, $J_{3,4}$ 10.6 Hz, H-3^{VI}), 4.05–3.90 (m, overlapped, H-2^{I}), 3.91 (t, partially overlapped, *J* 10.4, H-4^{VI}), 3.85 (dd, 1 H, $J_{2,3}$ 3.3 Hz, H-2^{VI}), 3.84 (m, 12 H, H-$4'^{I–VI}$a,b), 3.80–3.72 (m, 2 H, H-6″a,1″a), 3.60–3.56 (, 4 H, H-1″b, incl 3.57, s, OCH_3-1^{I}), 3.49 (m, 2 H, H-7″a,b), 2.31 (m, 2 H, H-5″a,b), 2.16–1.90 (2 m, 12 H, H-$3'^{I–VI}$a,b), 1.70–1.59 (m, 4 H, H-4″a,b,2″a,b), 1.43–1.35 (m, 2 H, H-3″a,b), 1.28–1.25 (m, 15 H, H-$6^{I–V}$), 1.23 (d, 3 H, $J_{5,6}$ 6.2 Hz, H-6^{VI}). Definitive signals in the ^{13}C NMR spectrum (D_2O) were at: δ 103.67, 103.61, 103.58, 103.56 (C-$1^{II–V}$), 101.81 (C-1^{VI}), 101.30 (C-1^{I}), 81.81 (C-2^{VI}), 80.53 (C-2^{I}), 80.39, 80.80 (2 C), 80.04 (C-$2^{II–V}$), 71.90 (C-$2'^{I–VI}$), 70.66 (C-1″), 63.84 (OCH_3-squarate), 61.63 (OCH_3-2^{VI}), 56.04, 55.99, 55.85 (2 C), 55.81 (2 C), (C-$4^{I–VI}$), 46.98 (C-6″), 42.09 (C-7″), 38.89 (3 C), 38.87 (2 C), 38.83 (C-$3'^{I–VI}$), 38.58 (C-5″), 31.12 (C-4″), 27.98 (C-2″), 27.78 (C-3″), 19.80, 19.74 (2C), 19.73, 19.70, 19.68 (C-$6^{I–VI}$). ESI-TOF-MS calculated for $C_{74}H_{125}N_8O_{41}$ $[M]^+$: 1780.7864, found: 1780.8054.

Methyl squarate derivative **13** A solution of amine **12** (10.5 mg, 0.01 mmol) and methyl squarate (2.1 mg, 0.015 mmol) in borate buffer (pH 7, 0.5 M, 0.5 mL) was stirred at room temperature overnight, when TLC (8:1 MeOH–H_2O) showed almost complete conversion of the starting amine material to a strongly UV positive product [17]. The mixture was applied directly on top of a 4 g SP tube and eluted with H_2O (8 mL), followed by a gradient H_2O→50% MeOH. Excess dimethyl squarate reagent (UV positive, non charring) was eluted first, followed by the desired **13**. Fractions containing the desired squaric acid derivative were freeze-dried, to give **13** (8 mg, 70%). ESI-TOF-MS calculated for $C_{49}H_{79}N_9NaO_{24}$ $[M+Na]^+$: 1200.5136, found: 1200.5140.

General Procedure for Conjugation of Hexasaccharide fragments of the O-PS of Vibrio cholerae *O:1 (serotypes inaba and ogawa*, ***8*** *and* ***11****) and the Peptidoglycan Disaccharide Tetrapeptide Fragment* ***13*** *to* ***rTT-H$_C$***. The hapten (**8**, **11**, **13**) was added to a solution of **rTT-H$_C$** in borate buffer (0.50 M, pH9.00) to make a 4 mM solution with respect to the hapten. The mixture was gently stirred at room temperature and the reaction was monitored periodically with SELDI-TOF MS, wherein either Sinapinic Acid or Ferulic acid served as the energy-absorbing molecule (EAM). When the desired carbohydrate-protein ratio was reached, as verified by SELDI-TOF MS, the reaction was terminated by the addition of ~2 mL of phosphate buffer (0.05 M, pH7.00). The mixture was transferred to a centrifugal filter device (10 k Amicon Ultra, Millipore) and dialyzed against 10 mM aqueous ammonium carbonate (eight times) to remove the low molecular mass material. The retentate was lyophilized to afford the conjugates (**A**, **B**, **C**, **D**) as white solids.

Conjugate A. This compound was obtained in 94% yield (16.5 mg) from the conjugation of **11** (3.1 mg, 0.0017 mmol) to **rTT-H$_C$** (15.0 mg, 0.00029 mmol). The SELDI-TOF MS analysis of **A** (Sinapinic Acid was used as the EAM) showed a **11**:**rTT-H$_C$** ratio of 4.9:1.0; loading efficiency, 82%.

Conjugate B. This compound was obtained in 94% yield (33.2 mg) from the conjugation of **8** (11.03 mg, 0.0062 mmol) to **rTT-H$_C$** (30.0 mg, 0.00058 mmol). The SELDI-TOF MS analysis of **B** (Ferulic Acid was used as EAM) showed a **8**:**rTT-H$_C$** ratio of 5.1:1.0; loading efficiency, 46%.

Conjugate C. This bivalent conjugate was obtained in 86% yield (22.2 mg) from the conjugation of an equimolar mixture of **8** and **11** (6.78 mg, 0.0038 mmol) to **rTT-H$_C$** (22.00 mg, 0.00042 mmol). The SELDI-TOF MS analysis of **C** (Sinapinic Acid was used as EAM) showed a carbohydrate:**rTT-H$_C$** ratio of 5.0:1.0; loading efficiency, 56%.

Conjugate D. This conjugate was obtained in two steps. In the initial step, hapten **11** (5.17 mg, 0.0029 mmol) was conjugated with **rTT-H$_C$** (25.0 mg, 0.00048 mmol) as described for preparation of **A**, to give an intermediate (27.0 mg, 91% yield; loading efficiency, 90%) similar to **A**, except for having a **8**:**rTT-H$_C$** ratio of 5.4:1. It was dissolved in borate buffer (0.5 M, pH9.00), to make a 2 mM solution with respect to **13**, and conjugated with **13** (3.09 mg, 0.0026 mmol). The SELDI-TOF MS analysis (Ferulic acid was used as EAM) of **D** (25.0 mg, 88% yield), gave a **11**:**13**:**rTT-H$_C$** ratio of 5.4:2.6:1.0; loading efficiency 43%.

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