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Design, Synthesis, and Immunological Evaluation of a Multicomponent Construct Based on a Glycotripeptoid Core Comprising B and T Cell Epitopes and a Toll-like Receptor 7 Agonist That Elicits Potent Immune Responses

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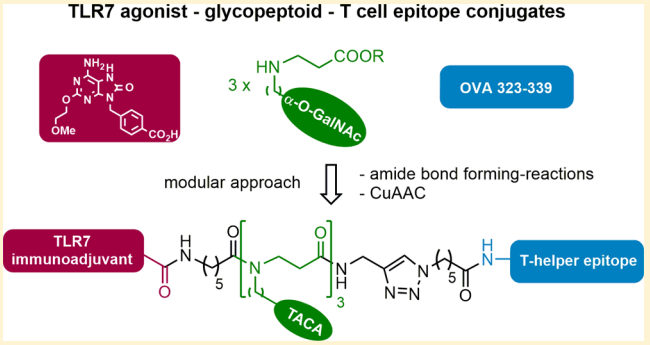
Supporting Information

ABSTRACT: We present here for the first time the synthesis and immunological evaluation of a fully synthetic three-component anticancer vaccine candidate that consists of a β-glycotripeptoid core mimicking a cluster of Tn at the surface of tumor cells (B epitope), conjugated to the OVA 323−339 peptide (T-cell epitope) and a Toll-like receptor 7 (TLR7) agonist for potent adjuvanticity. The immunological evaluation of this construct and of precursor components demonstrated the synergistic activity of the components within the conjugate to stimulate innate and adaptive immune cells (DCs, T-helper, and B-cells). Surprisingly, immunization of mice with the tricomponent GalNAc-based construct elicited a low level of anti-Tn IgG but elicited a very high level of antibodies that recognize the TLR7 agonist. This finding could represent a potential vaccine therapeutic approach for the treatment of some autoimmune diseases such as lupus.



INTRODUCTION

Anticancer immunotherapy represents a powerful new strategy in medicine. In particular, the administration of cancer vaccines[1] to harness the capacity of patient's immune system to eliminate tumor cells could represent an efficient alternative to usual antitumor treatments which are often unselective, accompanied by many side effects, and which may have difficulties in eradicating metastatic cells.[2]

Malignant transformations of normal cells are characterized by the overexpression of altered mucin-type glycoproteins or glycolipids. Aberrant glycosylations have been shown to contribute to the development of clustered tumor-associated carbohydrate antigens (TACAs),[3] such as (1) the glycoprotein antigens including the Tn (T nouvelle), Thomsen−Friedenreich (TF), and sialyl Tn (sTn) linked to serine or threonine residues and (2) glycolipid antigens including the gangliosides, the globo class, and the blood group determinants. TACAs represent attractive targets for antibody-mediated anticancer vaccines; however, as they are regarded as "autoantigens" or "self-antigens", they cannot alone activate helper T cells. Therefore, they exhibit weak immunogenicity, leading to difficulties in providing high affinity IgG to eliminate TACA-expressing human tumor cells. To circumvent these issues, intensive research has focused on the design and synthesis of a first generation of vaccine prototypes in which TACAs were conjugated to a foreign carrier protein (e.g., KLH or BSA) and used with a nonspecific immunoadjuvant to enhance helper T cell responses.[4] The notable contributions of Livingston and Danishefsky are key milestones in this research area. Particularly, based on the demonstration that Tn antigens are exposed on adjacent Ser/Thr residues in repeating clusters of 3−5, monovalent clustered vaccine candidates, wherein a mucin-derived glycopeptide-KLH conjugate contained three Tn antigens, have been developed.[5] These constructs, coadministrated with the QS-21 adjuvant, advanced to phase I clinical trials against prostate cancer and proved to elicit significant antibody responses in patients.[6] Nevertheless, in most cases, the structural heterogeneity of this generation of vaccine candidates and the potential immunogenicity of carrier proteins and linkers[7] resulted in suboptimal antibody responses against the TACA epitope, a critical issue for therapeutic efficacy of these cancer vaccines.

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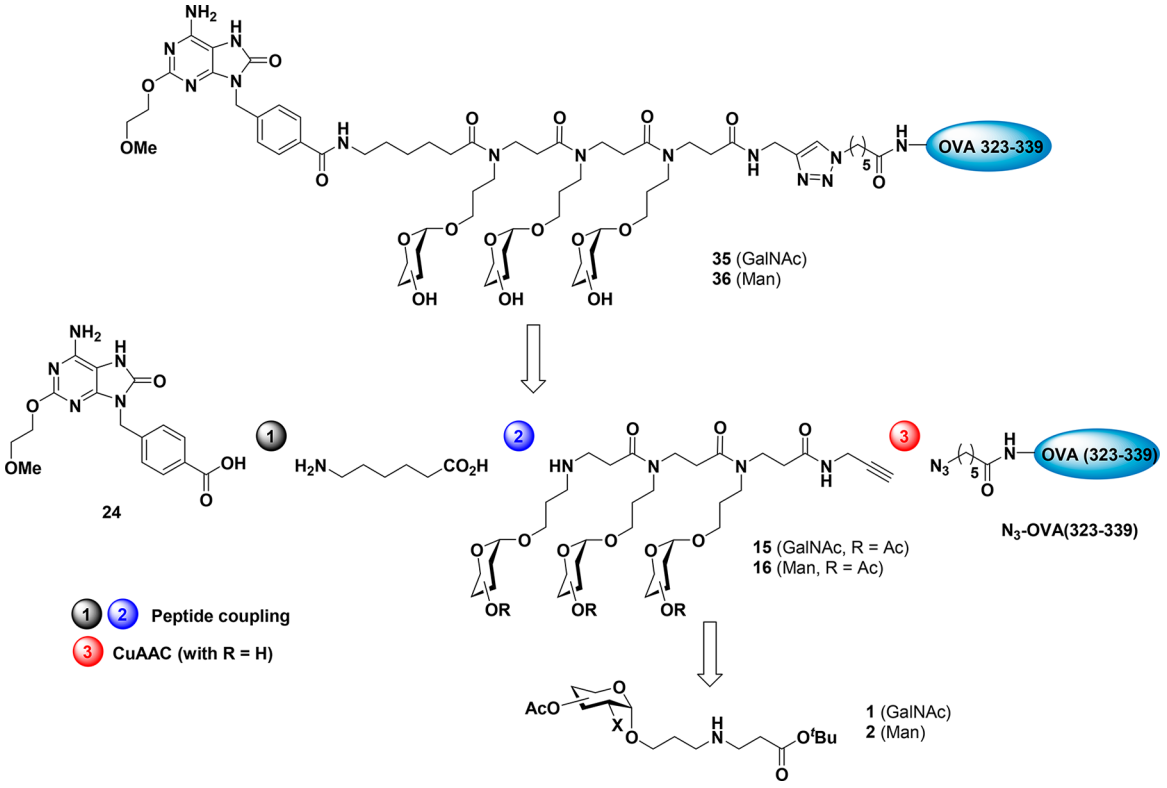

Figure 1. Strategy for the synthesis of the three-component carbohydrate-based constructs **35** and **36**.

With the aim of improving the presentation of the carbohydrate antigens in the absence of a carrier protein, a number of laboratories have designed fully synthetic multicomponent vaccines.[8−11] Considerable attention has been paid to the choice of scaffolds for the presentation of clustered antigenic units. Calix[4]arene,[12] cyclic decapeptides (RAFTs),[13,14] or dendrimeric polylysine fragments extended with glycopeptidic arms (MAG-Tn_3) have been chosen to develop multivalent vaccines.[15] The last, designed by the groups of Leclerc and Bay, exhibited four arms, composed of a $CD4^+$ peptide T-helper epitope and a trimeric Tn cluster. In the presence of a mild adjuvant, this construction elicited high level of anti-Tn IgG antibodies which have the ability to mediate the killing of Tn-positive tumor cells in mice[15] and non-human primates.[16] They also demonstrated that targeting macrophage galactose-type C-type lectins (MGL) by Tn tumor-associated antigens constitutes a promising approach for enhancing vaccine effectiveness.[17]

Moreover, advances in the knowledge of the coordination of innate and adaptive immune systems also offer new avenues for the design of anticancer vaccines. Indeed, the innate system detects large classes of highly conserved molecules that are integral parts of pathogens or abnormal cells. Their recognition through a set of pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs),[18] provides appropriate "danger signals" for the maturation of dendritic cells, which are fundamental targets for many clinical situations.[19−21] In this respect, the resulting production of proinflammatory cytokines influences innate and adaptive responses.[22,23] Some of these immune mediators are crucial for the differentiation of helper T cells toward a Th-1 or Th-2 phenotype. Efforts have thus been made to develop conjugates of tumor antigens and TLR ligands.[24−26] The Boons[27] and Payne[28] groups have for example developed multicomponent glycosylated MUC-1 based vaccine candidates incorporating a T-cell helper peptide and an immunostimulating lipopeptide TLR2 ligand. These vaccines presented remarkably high immunogenicity establishing the importance of TLR signaling for optimal immune responses.

Herein, following a similar approach, we report the synthesis and immunological evaluation of a structurally well-defined, fully synthetic three-component construct **35** that combines all the features required for a focused and effective activation of DCs, T- and B-cells and to elicit relevant IgG immune responses (Figure 1). This construct is composed of an original glycotripeptoid core with α-O-linked GalNAc moieties (B epitope). To strengthen its presentation to the immune system and to overcome the acquired immunotolerance to TACAs and their inherently T-cell independent nature, the glycopeptoid trimer was conjugated to the OVA 323−339 peptide (T_H epitope) and to a Toll-like receptor 7 (TLR7) heterocyclic agonist (immunoadjuvant).[29] The intracellular TLR7s act as innate immune sensors of microbial nucleic acids.[30]TLR7 (and TLR9) have also been proven to be implicated in the development of some autoimmune diseases, such as systemic lupus erythematosus (SLE),[31] as a consequence of the aberrant transportation of self-derived nucleic acids. Cell surface TLR7s were also recently revealed on bone marrow-derived macrophages.[32] As TLR7-dependent signaling is believed to promote autoimmune pathologies, constructs incorporating a TLR7 agonist can also be useful tools with respect to this type of disease. As immunoadjuvant, the major benefit of TLR7 agonists is their capacity to stimulate several cell types (APCs, CD4 and CD8 T cells, and NK cells) resulting in a broad spectrum of activated immune cells, cytokines, and chemokines at the tumor site.[33] A tricomponent construct analogue (**36**) in which the three GalNac were replaced by three mannoses, was also synthesized

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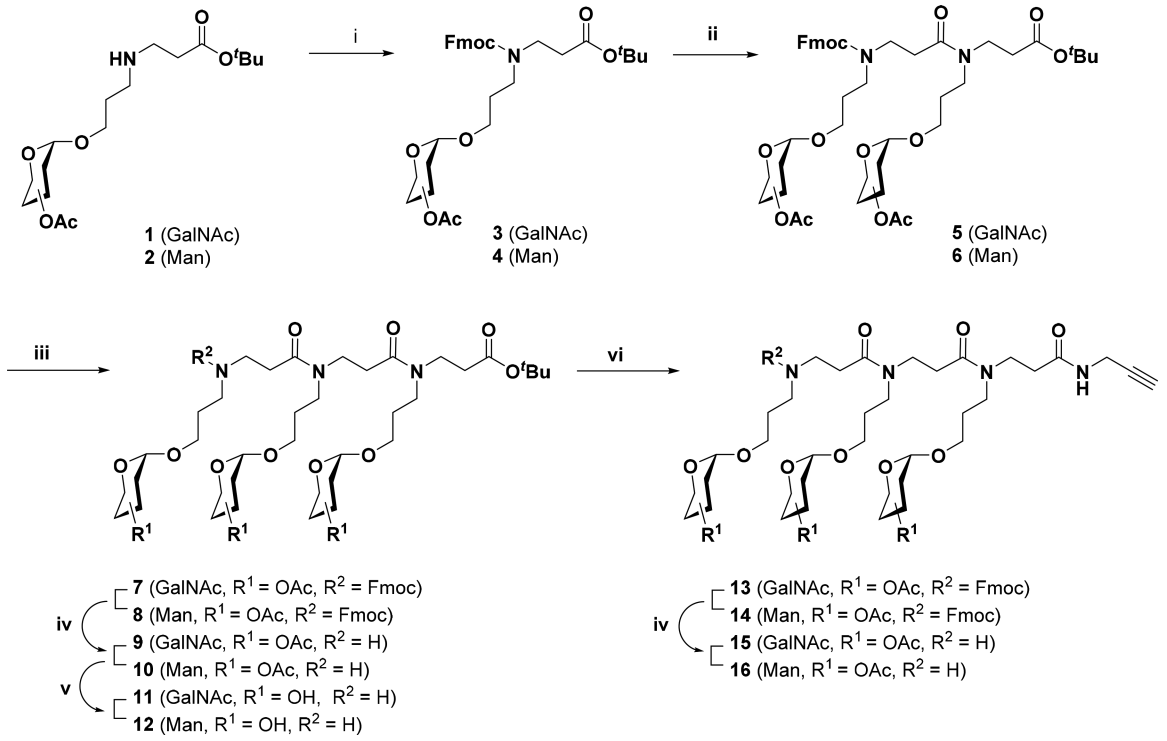
Scheme 1. Synthesis of the C-Terminus Alkyne-Functionalized β-Glycotripeptoids 15 and 16[a]



[a]Reagents and conditions: (i) Fmoc-Cl, $NaHCO_3$, 1,4-dioxane, rt, 4 h, 84% (**3**), 91% (**4**); (ii) TFA, CH_2Cl_2 (1:1, v/v), rt, 1 h, quantitative, then **1** or **2**, HATU, DIEA, CH_2Cl_2, DMF, rt, overnight, 81% (**5** from **1** and **3**), 90% (**6** from **2** and **4**); (iii) TFA, CH_2Cl_2 (1:1, v/v), rt, 1 h, quantitative, then **1** or **2**, HATU, DIEA, CH_2Cl_2, DMF, rt, overnight, 75% (**7** from **1** and **5**), 87% (**8** from **2** and **6**); (iv) Et_2NH, CH_3CN (1:2, v/v), rt, 4 h, quantitative; (v) NaOMe, MeOH, rt, 1 h, quantitative; (vi) **7** or **8**, TFA, CH_2Cl_2 (1:1, v/v), rt, 1 h, quantitative then H_2NCH_2CCH, HATU, DIEA, CH_2Cl_2, CH_3CN, rt, overnight, 95% (**13**), 86% (**14**).

as a negative control with respect to the production of anti-Tn IgG. A β-peptoid platform was chosen for bringing together all the components of the constructs **35** and **36**. β-Peptoids (N-substituted β-alanine oligomers) are a class of homologous peptoids,[34,35] being defined as N-substituted glycine oligomers.[36,37] In other words, what distinguishes peptoids from peptides is the fact that the side chains are located on the nitrogen atoms of the backbone amides instead of being attached to the main chain carbons atoms. This structural change confers advantages over peptides, notably an enhanced metabolic stability[38,39] and a greater cell-permeability,[40−42] two important factors for their use as peptidomimetics or chemical platforms. The presence of peptoid tertiary amide bonds, prone to cis/trans isomerism,[43−45] is also responsible for a greater backbone flexibility. Different strategies aimed at promoting either the cis- or trans-amide bond conformations have been developed in the recent past,[46−48] notably by our group.[49−53] These strategies might be harnessed in the future to reduce flexibility of peptoid-based vaccine constructs. For the initial design, we have selected a conformationally flexible glycopeptoid moiety, assuming that this design will increase the binding of the constructs to C-type lectin receptors (CLRs) at the surface of DCs. In addition, the flexibility of B cell epitopes plays a major role in their capacity to induce antibody responses.[54] It is also for that reason that we chose a β-peptoid backbone rather than the more common α-peptoid backbone.[55]

To date, this is the first time that a peptidomimetic structure and a TLR7 adjuvant were used and attached together in an anticancer vaccine prototype. The adjuvanticity and the T-antigenicity of the construct were first investigated in vitro by ELISA and T-cell stimulation assays, respectively. Its immunogenicity, i.e., the ability to elicit anti-Tn IgG, was then analyzed in vivo in mice. The synthetic and biological results are reported in detail in this paper.

RESULTS AND DISCUSSION

Design of the Three-Component Constructs 35 and 36 Based on a TLR7 Agonist and Multivalent B/CD4$^+$ T-Cell Epitopes. We developed a convergent solution-phase synthetic approach for the construction of the synthetic targets **35** and **36**, based on two amide bond forming reactions and a chemoselective copper azide−alkyne cycloaddtion (CuAAC) reaction (Figure 1). The synthetic targets were composed of four modules that were assembled sequentially: the TLR7 agonist **24** equipped with a carboxylic acid function, a 6-aminocaproyl group used as a spacer, the glycopeptoid cores **15** and **16** ended by a propargyl amide function, and the OVA 323−339 peptide bearing a 6-azidohexanoyl arm. In this modular approach, the TLR7 agonist **24** was first coupled to the aminocaproic acid spacer, which in turn was coupled to the N-terminus of the glycopeptoid cores. Finally, the resulting constructs were conjugated to the OVA peptide by a CuAAC reaction.

Two approaches were envisaged to access the conformationally flexible glycopeptoids[56] cores **15** and **16**: a monomer approach consisting of the peptide coupling of orthogonally protected glycosylated β-alanine monomers and the so-called submonomer approach initially developed for the synthesis of α-peptoid oligomers[36] and adapted for β-peptoid synthesis.[34]

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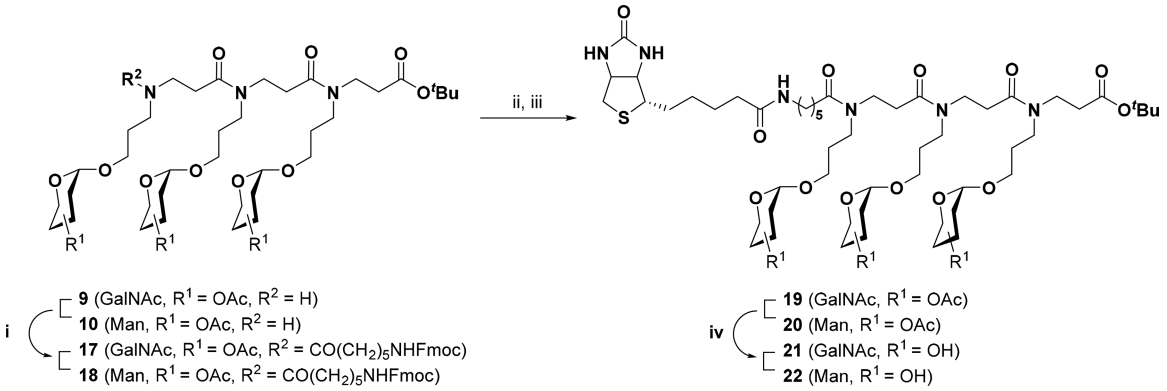
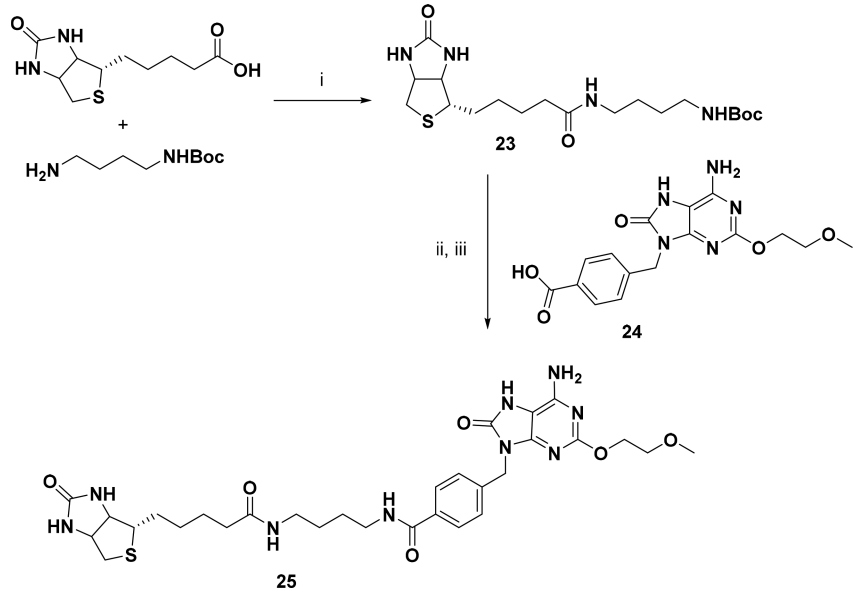
Scheme 2. Biotinylation of Glycopeptoid Platforms 9 and 10[a]



[a]Reagents and conditions: (i) **9** or **10**, $HO_2C(CH_2)_5NH$-Fmoc, HATU, DIEA, CH_2Cl_2, DMF, rt, 14 h, 50% (**9**), 90% (**10**); (ii) **17** or **18**, Et_2NH, CH_3CN (1:2, v/v), rt, 4 h; (iii) biotin *N*-hydroxysuccinimide, Et_3N, DMF, rt, 16 h, 81% (**17**), 37% (**18**); (iv) NaOMe, MeOH, rt, 1 h, quantitative.

Scheme 3. Biotinylation of TLR7 Agonist 24[a]



[a]Reagents and conditions: (i) biotin (1 equiv), IBCF 1.2 equiv, Bu_3N 1.3 equiv., DMF, rt, then 0 °C, $BocNH(CH_2)_4NH_2$ in DMF, 4h, rt; (ii) TFA/DCM 1/1; (iii) HATU 2 equiv, DIEA 5 equiv, **24** (1 equiv) DMF.

The submonomer synthesis consists of creating each new monomer in two steps, which are, in the case of β-peptoid synthesis, acylation of the N-terminus of the growing chain with acryloyl chloride followed by the aza-Michael addition of a primary amine to the formed acrylamide system. It is known that this two-step methodology is not as effective as the submonomer synthesis of α-peptoids,[57] but it could reasonably be considered for the synthesis of the short glycosyl-β-peptoid trimers **15** and **16**. Both monomer and submonomer methodologies entailed the synthesis of the peracetylated building blocks **1** and **2** (Scheme 1, Supporting Information).[58,59] The solution-phase submonomer synthesis of β-peptoids has been optimized in our group, notably when volatile amines are used in the Michael addition steps.[60,61] Application of our optimized protocol to form β-glycopeptoids trimers was however unsuccessful. We subsequently turned to the use of the "monomer method" for preparing trimers **15** and **16**.

The adenine-like heterocyclic moiety **24** is a potent immunoadjuvant eliciting its activity through the interaction with Toll-like receptor 7.[29] This leads to maturation of DCs and production of cytokines which promote the development and activation of T helper cells. Moreover, its pharmacological potency is improved after conjugation to chemical entities that facilitate endocytosis.[62,63] Aminocaproic acid was used as a spacer between the TLR7 agonist **24** and glycopeptoid components. The choice of the peptide fragment OVA 323−339 (ISQAVHAAHAEINEAGR) from ovalbumin protein was guided by past work showing that it contains multiple T helper epitopes involved in high affinity humoral responses.[64−67] The CuAAC was envisioned as a convenient strategy for chemoselective assembling of OVA 323−339 to the TLR7-glycopeptoid conjugates.[68,69] Indeed, this very popular ligation method allows in a single step for the formation of an amide bioisostere[70−72] between unprotected fragments and is compatible with a plethora of chemical functions. In this respect, the OVA 323−339 moiety was purchased with an azide-functionalized arm at the N-terminus and the glycoconjugates **15** and **16** were engineered with a C-terminal alkyne function.

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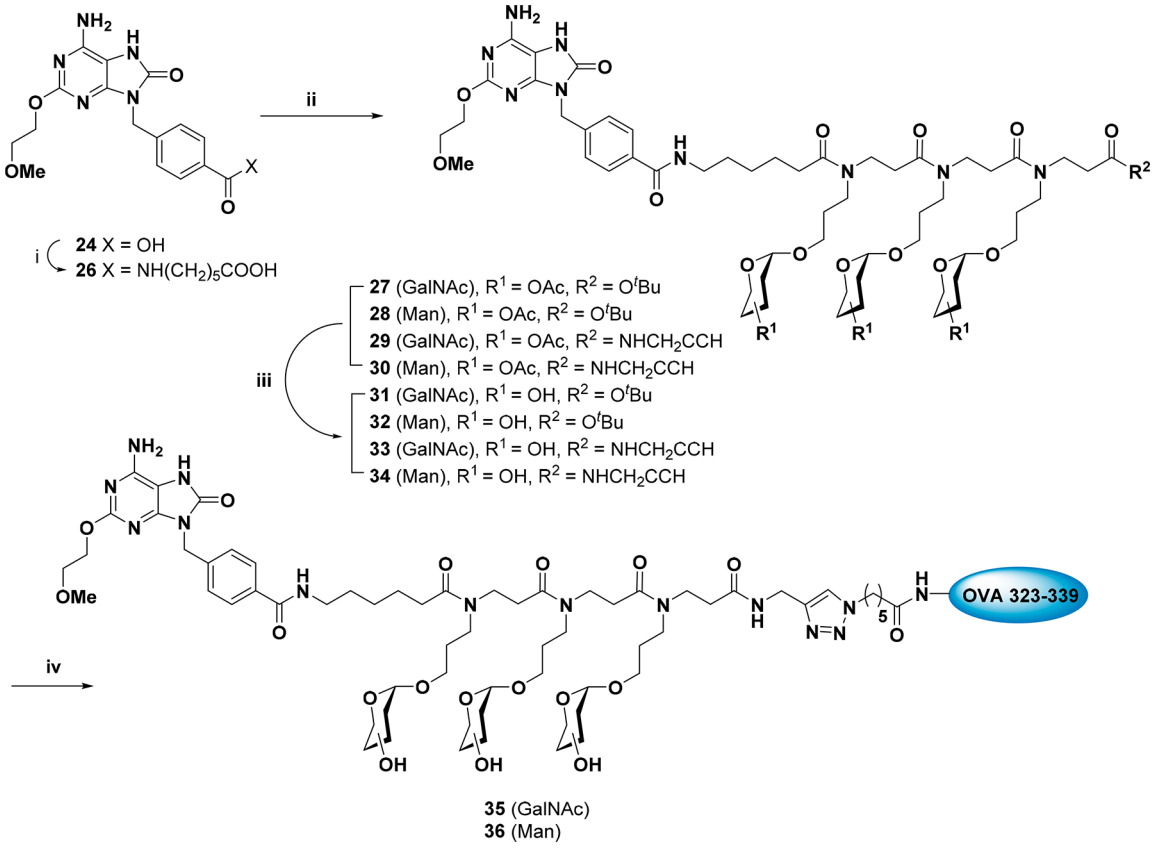
Scheme 4. Synthesis of the Three-Component Carbohydrate-Based Constructs[a]



[a]Reagents and conditions: (i) aminocaproic acid, IBCF, $N(Bu)_3$, DMF, H_2O (3:1, v/v), 0 °C, 24 h, 55%; (ii) **9** or **10** or **15** or **16**, HATU, DIEA, CH_2Cl_2, DMF, rt, overnight, 53% (**27** from **9**), 53% (**28** from **10**), 48% (**29** from **15**), 50% (**30** from **16**); (iii) NaOMe, MeOH, rt, 1 h, quantitative; (iv) N_3-OVA 323−339, THPTA, aminoguanidine hydrochloride, HEPES buffer (100 mM, pH 7.5), H_2O, HFIP, 1−3 h, 80% (**35** from **33**), 77% (**36** from **34**).

Synthesis of the Core Glycopeptoids 15 and 16. Various strategies have been developed for synthesizing glycopeptoids including postmodifications of functionalized side chains for the anchoring of carbohydrate moieties.[73] A "submonomer" approach has been adapted for the synthesis of *β*-peptoid oligomers in which the side chains are incorporated by aza-Michael addition to acrylamide intermediates.[34] In the present work, the strategy that has proved the most effective in synthesizing the core glycopeptoids **15** and **16** is the peptide coupling of the synthesized N-substituted *β*-alanine monomers **1** and **2** (Scheme 1). They were prepared in six steps from D-GalNAc and D-mannose, respectively (Supporting Information). First, monomers **1** and **2** were protected as Fmoc carbamates by treatment with Fmoc-Cl and saturated $NaHCO_3$ in dioxane to provide **3** (84%) and **4** (91%), respectively. With these orthogonally protected monomers in hand, the construction of trimers **15** and **16** was then accomplished using an iterative sequence, consisting of forming the free acid partners and their subsequent coupling with the amine **1** or **2**. From monomers **3** and **4**, the corresponding free carboxylic acids were quantitatively produced by hydrolysis of the ^{t}Bu esters (TFA/CH_2Cl_2, 1:1, v/v) and coupled with their amine partner (**1** or **2**) with HATU/DIEA-optimized conditions (CH_2Cl_2/DMF (7:3, v/v)). Glycopeptoids dimers **5** and **6** were isolated in 81% and 90%, respectively, after silica gel column chromatography. Reiteration of the deprotection−coupling process furnished glycotripeptoids **7** and **8** in 75% and 87%, respectively. One portion of each of them was further deprotected at the N-terminus (Et_2NH/CH_3CN, 1:2, v/v) followed by Zemplén deacetylation of the carbohydrate moieties for biological testing (compounds **11** and **12**).

As the strategy for conjugation of the core glycopeptoid trimers **7** and **8** with the OVA 323−339 peptide was based on the non-native CuAAC reaction, it was therefore necessary to equip the C-termini of trimers **7** and **8** with a terminal alkyne arm. Accordingly, the two trimers were treated with TFA/CH_2Cl_2 (1:1, v/v) for removal of the ^{t}Bu esters, followed by a peptide coupling with propargylamine by employing HATU-DIEA conditions to give **13** (95%) and **14** (86%). **13** and **14** were further deprotected at the N-terminus (Et_2NH/CH_3CN, 1:2, v/v) to yield compounds **15** and **16** ready for conjugation with the TLR7 agonist moiety **26**.

At this stage of the synthetic work, the TLR7 agonist (**24**) and glycopeptoids components (**9**, **10**) were biotinylated for enzyme-linked immunosorbent assay (ELISA) aimed at analyzing the specificity of the antibodies induced by the constructs (Schemes 2, 3).

Conjugation to the TLR7 Agonist and OVA 323−339 Peptide. The TLR7 heterocyclic agonist **24**, chosen in the present work, was prepared from xanthine following adaptations of published procedures (Supporting Information).[29] The TLR7 heterocyclic agonist **24** was first coupled to aminocaproic acid, used as a spacer, employing IBCF and

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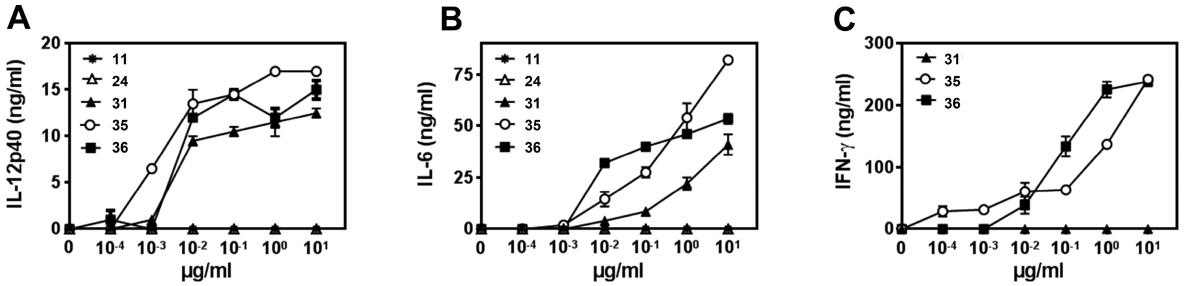

Figure 2. In vitro adjuvanticity and T-antigenicity of the glycoconjugates **31**, **35**, and **36**. Mouse BMDCs (10^5) were cultured at 37 °C either with increasing concentrations of **11**, **24**, **31**, **35**, or **36** for 48 h or with increasing concentrations of the same compounds for 3 days in the presence of OT-II CD4$^+$ T lymphocytes isolated from OT-II transgenic mice. IL-12p40 (A), IL-6 (B), and IFN-γ (C) were measured in cell supernatants by ELISA.

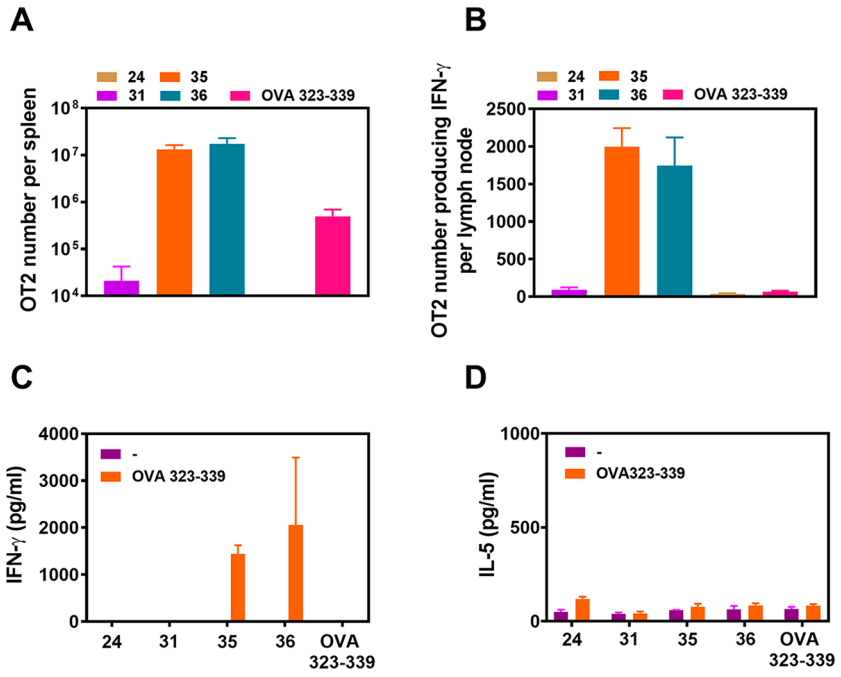

Figure 3. In vivo T-immunogenicity of the three-component glycopeptoids **35** and **36**. After administration of 2 × 10^5 OT-II CD4$^+$ T cells, C57BL/6 mice (n = 3 per group) were intradermally immunized with **24**, **31**, **35**, **36**, or OVA 323−339 (11.7 nmol per mouse). After 5 days, mice were sacrificed and then the spleen and draining lymph node were collected. OT-II CD4$^+$ T cells proliferation (A) was detected by flow cytometry using FITC labeled antibodies anti-CD4, anti-Vα2, and anti-Vβ5. Among CD4$^+$ population, the number of OT-II CD4$^+$ T cells in lymph node that produce IFN-γ (B) was determined using FITC labeled antibody anti-IFN-γ. The splenocytes were cultured (5 × 10^5) in the presence or not of OVA 323−339 (1 μg/mL) at 37 °C for 48 h, and IFN-γ (C) and IL-5 (D) levels were measured in the supernatants by ELISA.

$N(Bu)_3$ in DMF/H_2O (3:1, v/v) (Scheme 4). The formed compound **26** was then conjugated to the glycotripeptoid scaffolds **9**, **10**, **15**, and **16** to yield glycoconjugates **27** (53%), **28** (53%), **29** (48%), and **30** (50%), respectively. All of them were deacetylated, giving compounds **31**, **32**, **33**, and **34** in essentially quantitative yields and high purity.

The deacetylated conjugates **31** and **32** made of only two components of the three composing the final constructs (**35**, **36**) were intended to be subject to the immunological tests. On the other hand, the deacetylated oligomers **33** and **34** bearing an alkynyl group were intended to be used for the conjugation to the azide-functionalized OVA 323−339 peptide employing the CuAAC reaction. The OVA peptide was indeed purchased with a 6-azidohexanoyl arm at its N-terminus to allow the CuAAC to proceed. An air-free procedure developed by Finn et al.[74] was applied to avoid damage of the peptide due to copper-mediated production of reactive oxygen species. In addition to the $CuSO_4$/sodium ascorbate reagents, the THPTA copper(I) stabilizing ligand, and aminoguanidine hydrochloride, an additive known to intercept byproducts of ascorbate oxidation was also used.

The CuAAC ligations were conducted under an argon atmosphere, and all the solvents were carefully degassed. For the first attempt, **34** and the N_3-OVA 323−339 peptide were engaged (1:1 ratio, ∼5 mM) in a HFIP/HEPES buffer mixture at pH 7.5. The catalytic solution was composed of $CuSO_4$ (2 equiv), sodium ascorbate (4 equiv), THPTA (6 equiv), and aminoguanidine (5 equiv). Under these conditions, **34** and OVA were not totally consumed after 1 h. Another catalytic solution was added with the same amount of each compound except for $CuSO_4$ (4 equiv) and sodium ascorbate (8 equiv). After 2 h of reaction, $CuSO_4$ and sodium ascorbate were added again. Finally, a complete conversion was observed within 3 h and a C18 SPE cartridge purification afforded the pure O-mannosylated three-component construct **36** in a good 77% yield. In order to reduce the reaction time between **33** and N_3-

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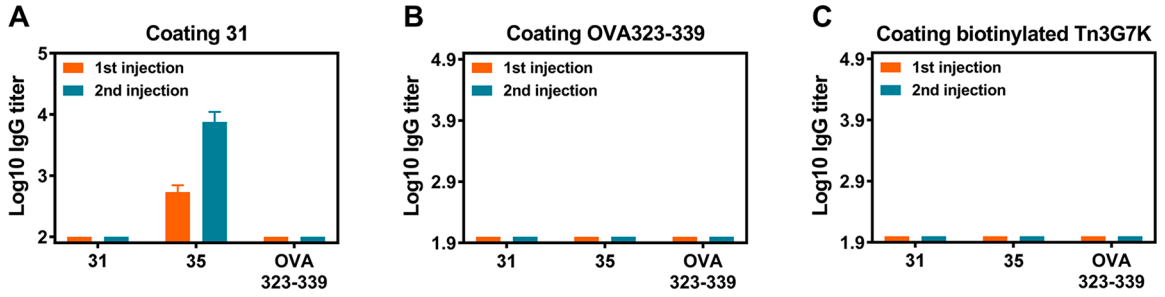

Figure 4. In vivo B-immunogenicity of the three-component glycopeptoids **35**. C57BL/6 mice (n = 3 per group) were intradermally immunized on days 0 and 21 with 11.7 nmol of **31**, **35**, or OVA 323−339. Sera were collected on day 0 and before (day 20) or after (day 28) the second injection and were tested for IgG antibodies by ELISA using **31**, OVA 323−339, or biotinylated Tn3G7K coated at 1 μg/mL.

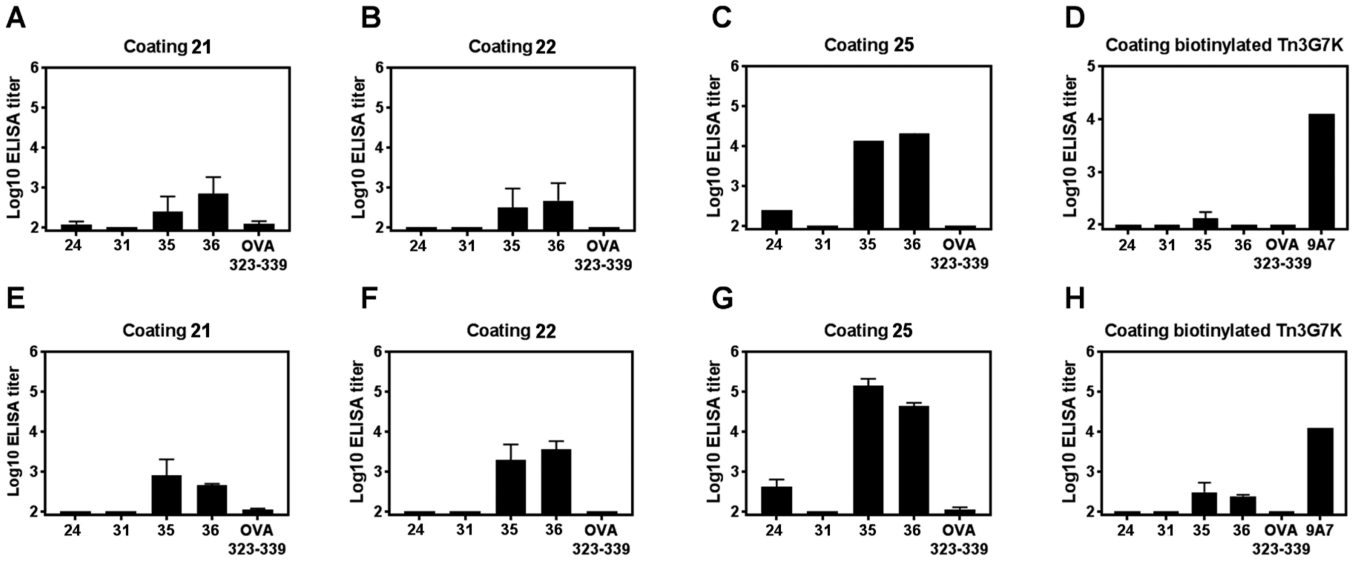

Figure 5. Superior efficacy of the three-component glycopeptoids **35** and **36** in their ability to induce anti-TLR7 agonist IgG. (A−H) C57BL/6 mice (n = 3 per group) were intradermally immunized on days 1 and 20 and then intravenously on day 92 with 11.7 nmol of the compounds **24**, **31**, **35**, **36** or the peptide OVA 323−339. Sera were collected after the second (A−D, day 27) and the third (E−H, day 111) injections and were analyzed for IgG antibodies by ELISA using **21**, **22**, **25**, or biotinylated Tn3G7K coated at 1 μg/mL.

OVA 323−339, we envisioned directly introducing a catalytic solution with more significant amounts of $CuSO_4$ (4 equiv), sodium ascorbate (8 equiv), and aminoguanidine (10 equiv) but with the same amount of THPTA. Hence, a complete conversion was obtained within 1 h. After a C18 SPE cartridge purification, the *O*-GalNAc three-component construct **35** was provided in high purity and in good 80% yield.

Immunology. We first evaluated in vitro the capacity of the constructs to retain the immunological properties of the original components, through the analysis of the adjuvant activity of the TLR7 ligand and the T cell antigenicity of the OVA 323−339 peptide. The adjuvant property of the various constructs was analyzed for their capacity to trigger the production of cytokines by murine DC (Figure 2). Compounds **31**, **35**, and **36**, containing a TLR7 agonist, induced strong and comparable IL-12 and IL-6 secretion (Figure 2A and Figure 2B). In contrast, the GalNAc glycopeptoid trimer **11** and TLR7 agonist **24** alone were unable to induce the production of cytokines. Taken together, these results confirm the functional role of the TLR7 agonist moiety to stimulate mouse BMDCs and highlight the importance of the conjugation of this TLR7 ligand to the glycopeptoid moiety for the activation of the TLR7 endosomal receptors. Recruitment of MGL receptors is likely to promote DC activation.[75] Further, it has been documented that the immunostimulatory activity of a TLR7 ligand can be amplified by conjugation.[29] We next tested the IFN-γ production by OVA-specific OT-II T cells in response to the constructs (Figure 2C). Compounds **35** and **36**, which contain the OVA 323−339 peptide but not **31**, potently activated OT-II T cells in the presence of DCs.

The in vivo immunogenicity of the constructs was then evaluated. C57BL/6 mice received OT-II cells specific for the OVA 323−339 peptide and were then immunized with compounds **24**, **31**, **35**, **36** or with the OVA 323−339 peptide. Five days later, we analyzed the OT-II T cell responses in spleen and lymph nodes (LN). In the spleen of mice immunized with the compounds **35** and **36**, we detected 30-fold more OT-II T cells, as compared to the mice immunized with the OVA 323−339 peptide (Figure 3A). These results showed that the TLR7 agonist, whatever the GalNAc or Man glycosylation, potently enhanced the activation of OT-II cells by the OVA T cell epitope, most likely through DC activation. Lymph node IFN-γ producing T cells were then analyzed by intracellular cytokine staining using flow cytometry (Figure 3B). In mice immunized with the OVA 323−339 peptide in the absence of adjuvant, no IFN-γ production was detected despite the proliferation of OT-II cells. In contrast, in mice

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immunized with compounds **35** and **36**, we observed a high and comparable IFN-γ production by LN OT-II T cells, demonstrating no major influence of the type of glycosylation. In vitro stimulation by the OVA peptide of the splenocytes of mice immunized with the compounds **35** and **36** induced the production of high level of IFN-γ but not of IL-5 (Figure 3C and Figure 3D), suggesting a Th1 phenotype of the activated T cells, in agreement with the capacity of these compounds to induce the production of IL-12 by DC (Figure 2A). Control **24** and **31** compounds, devoid of the OVA peptide, did not induce any T cell proliferation or activation (Figure 3, all panels).

We then assessed the antibody responses of mice immunized twice at days 0 and 21 with **31**, **35**, or peptide OVA 323−339 (Figure 4). No antibody response was detected against the OVA peptide or against the biotinylated Tn3G7K.[76] However, in the group immunized with compound **35**, high antibody responses were detected against compound **31**, demonstrating the B cell immunogenicity of the immunogen containing glycans, the peptoid moiety, and the TLR7 agonist (Figure 4).

To further investigate the fine specificity of the antibodies induced by the immunogen **35** and **36**, we analyzed their reactivity by ELISA against the compounds **21**, **22**, and **25**. For this purpose, the serum of mice immunized by the constructs **35** and **36** or by the controls **24** and **31** were collected and analyzed at days 0 and 21 for their capacity to recognize the glycan biotinylated Tn3G7K, the peptoid moiety **21** and **22** or the TR7 agonist **25** (Figure 5A−D). Mice also received a third injection of the compounds at day 92 (Figure 5E−H), and their sera were analyzed at day 111.

Both compounds **21** and **22** were recognized by the sera of mice immunized by **35** and **36**, showing that independent of the glycosidic part, the peptoid backbone is slightly immunogenic (Figure 5A,B,E,F). However, the recognition of the peptoid remained low as compared to the very high level of antibodies (up to 100 000) directed against the TLR7 agonist (Figure 5C and Figure 5G). Tn3G7K, expressing the Tn antigen, was not recognized by the sera of mice immunized by **35** and **36**, in contrast to the 9A7 monoclonal antibody used as a positive control, demonstrating that the antibody responses induced by these compounds are in large part directed against the TLR7 agonist (Figure 5D and Figure 5H)

Thus, in the present study, we have validated in vitro and in vivo the functionality and efficiency of the three conjugated components (TLR7 agonist, glycoclusters, and OVA 323−339). The novel synthetic constructs **35** and **36** are highly immunogenic but induce unexpected immunological responses. Indeed, instead of the production of IgG antibodies against the Tn trimeric cluster as observed with other synthetic constructs, these compounds elicited in mice low titers of anti-glycopeptoid (mannose and GalNAc) IgG but very high levels of IgG antibodies that recognize the TLR7 agonist. The lack of Tn reactivity probably reflects the absence of natural Ser/Thr amino acid backbone for the GalNAc within the constructs (Figure 4B and Figure 4F). As the TLR7 (and TLR9) have been implicated in the development of some autoimmune pathologies, such as systemic lupus erythematosus,[31,32] the vaccination against TLR7 agonists could represent a potential therapeutic approach for the treatment of lupus and other autoimmune diseases. Several compounds that bind to endosomal TLR7 and/or TLR9 are indeed being developed with the goal to inhibit IFN production and activation of autoimmune B cells.[77]Thus, circulating antibodies capable of capturing TLR7 activating molecules could represent an alternative strategy to inhibit TLR7 stimulation. The capacity of constructs to induce the production of antibodies against TLR7 agonists was never analyzed and thus clearly deserves further investigations.

CONCLUSION

We have designed, synthesized, and evaluated the three-component construct **35** as a novel potential anticancer vaccine candidate. In this respect, we have investigated the use of a β-peptoid scaffold for the preparation of this three-component construct. By sequential amide bond forming reactions and a CuAAC ligation, a β-glycotripeptoid molecule (B epitope), displaying clustered Tn antigen analogues to mimic the epithelial tumor surface, a TLR7 immunoadjuvant and an OVA 323−337 peptide (T-helper epitope) were conjugated together. To the best of our knowledge, this structure represents the first example of a self-adjuvanted vaccine candidate built on a β-peptoid platform. The immunological evaluation of our original multiepitopic glycoconjugate **35** clearly demonstrated an effective activation of innate and adaptive immune cells (DCs, T-helper, and B-cells), leading to robust IgG antibody responses. We can conclude from these results that compound **35** is highly immunogenic and that the functionality of the three components within the conjugate has been validated in vitro and in vivo. We have also demonstrated that the incorporation of a TLR7 agonist is crucial for the immunogenicity of the construct, providing the expected cytokines to induce maturation of DCs.

However, a surprising finding was that, instead of the production of IgG antibodies against the target Tn antigen, **35** elicited very high levels of IgG antibodies against the TLR7 agonist. Altogether, these preliminary studies could suggest that the organization of the Tn Ag analogs on the flexible β-peptoid scaffold does not provide a convenient clustered conformation for eliciting such antibodies responses. Alternatively, it could be suggested that the conformation of compound **35** is favorable for the presentation of the TLR7 agonist to specific B cells. This could be exploited for the induction of powerful antibody responses against circulating DNA. The TLR7 and TLR9 have indeed been implicated in the development of some autoimmune pathologies, such as systemic lupus erythematosus.[31,32] The vaccination against TLR7 agonists could represent a potential therapeutic approach for the treatment of lupus and other autoimmune diseases.

Several improvements must be made to the tricomponent vaccine candidate presented here to induce antibody responses against the Tn antigen. The modularity of our construct and the efficiency of the synthetic strategy have to be further exploited to improve the anti-Tn response from a novel design of glycopeptoids displaying clustered Tn Ags, possibly closer to the modified mucins comprising other heterocyclic TLR7 immunoadjuvants.

EXPERIMENTAL SECTION

Chemistry. Materials and Methods. All anhydrous reactions were performed under an argon atmosphere. CH_2Cl_2 was distilled from CaH_2 under argon before use. MeOH was distilled from MgI_2 under argon before use. THF was distilled from sodium/benzophenone under argon before use. DMF and CH_3CN were obtained from commercial sources and stored over 4 Å molecular

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sieves. EtOAc was distilled and stored over 4 Å molecular sieves. MeOH, EtOAc, cyclohexane, and CH_2Cl_2 for column chromatography were distilled before use. Et_3N was distilled from KOH and stored over 4 Å molecular sieves. All other solvents and chemicals were obtained from commercial sources and used as supplied. The N_3-OVA 323−339 peptide (immunograde purity) equipped with a 6-azidohexanoyl arm was purchased from Polypeptide Laboratories, France. TLC was performed on Merck TLC aluminum sheets, silica gel 60, F_{254}. The extent of reactions was, when applicable, followed by TLC and/or HPLC. Components were made visual with UV light and/or vanilline in $EtOH/H_2SO_4$ and/or 30% sulfuric acid in MeOH. Flash chromatography was performed with Merck silica gel 60, 40−63 μm. IR spectra were recorded on a Shimadzu FITR-8400S spectrometer equipped with a Pike Technologies MIRacle ATR, and wavenumbers (ν) are expressed in cm^{-1}. NMR spectra were recorded on a 400 MHz Bruker Avance 400 spetrometer. Chemical shifts, reported in δ ppm, are referenced to the residual solvent peak. The following multiplicity abbreviations are used: (s) singlet, (d) doublet, (t) triplet, (quint) quintuplet, (dd) doublet of doublets, (dt) doublet of triplets, (ddd) doublet of doublet of doublets, (m) multiplet. All NMR spectral data for glycopeptoids are of rotameric mixtures. HRMS were recorded on a Micromass Q-Tof Micro (3000V) apparatus. RP-HPLC analyses were performed on a Dionex instrument equipped with an Uptisphere strategy column (C18, 5 μm, 100 Å, 4.6 mm × 250 mm) and a UVD340U detector. All tested compounds had purity of ≥95% as determined by analytical HPLC.

***N*-Fmoc Protected *O*-GalNAc Glycoside 3.** Compound **1** (360 mg, 0.7 mmol) was suspended in 1,4-dioxane (1.4 mL) and saturated $NaHCO_3$ solution (3.7 mL) at 0 °C under an argon atmosphere. Then, Fmoc-Cl (350 mg, 1.3 mmol) in 1,4-dioxane (1 mL) was added and the mixture was stirred at room temperature for 4 h. The reaction was dissolved in AcOEt (30 mL). The organic layer was washed with H_2O (3 × 10 mL), dried ($MgSO_4$), and concentrated under reduced pressure. The crude was purified by column chromatography (AcOEt/cyclohexane, 95:5) to give compound **3** as a yellow oil (422 mg, 84%). RP-HPLC t_R, 20.5 min (91.7%) and 22.6 min (8.2%), $MeOH/H_2O$ 80:20, 99.9% purity, 210 nm. IR (ATR): 3353, 3323, 2979, 2927, 1747, 1728, 1700, 1695, 1679, 1560, 1529, 1479, 1458, 1451, 1369, 1224, 1221, 1153, 1131, 1075, 1044, 953, 739, 737 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 1.42 (m, 9H, *t*-Bu), 1.67−1.81 (m, 2H, $CH_2CH_2CH_2$), 1.93−2.19 (m, 13H, 3 × OAc, NHAc, 0.5 × $CH_2CO_2{}^tBu$), 2.45 (m, 1H, 0.5 × $CH_2CO_2{}^tBu$), 3.06−3.66 (m, 6H, 2 × CH_2-N, CH_2-O-GalNAc), 4.03−4.22 (m, 4H, $H_{5'}$, 2 × $H_{6'}$, CH Fmoc), 4.50 (m, 2H, CH_2 Fmoc), 4.60 (m, 1H, $H_{2'}$), 4.80 (m, 1H, $H_{1'}$), 5.13 (dd, 1H, J = 3.0, 11.7 Hz, $H_{3'}$), 5.37 (d, 1H, J = 3.0 Hz, $H_{4'}$), 6.19−6.79 (m, 1H, NHAc), 7.32 (t, 2H, J = 7.6 Hz, 2 × CH aromatic), 7.39 (t, 2H, J = 7.6 Hz, 2 × CH aromatic), 7.55 (m, 2H, 2 × CH aromatic), 7.75 (d, 2H, J = 7.6 Hz, 2 × CH aromatic). HRMS (ESI): calculated for $C_{39}H_{51}N_2O_{13}$ $[M + H]^+$, 755.3391; found, 755.3402.

***N*-Fmoc Protected O-Mannosylated Glycoside 4.** A procedure similar to that for compound **3** was used to obtain compound **4** as a yellow oil (535 mg, 91%) from **2**. RP-HPLC t_R, 26.1 min, $MeOH/H_2O$ 80:20, 100% purity, 214 nm. IR (ATR): 2964, 2939, 2920, 1747, 1701, 1479, 1450, 1423, 1368, 1220, 1151, 1136, 1084, 1047, 980, 760, 742 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 1.43 (m, 9H, *t*-Bu), 1.50−1.80 (m, 2H, $CH_2CH_2CH_2$), 1.98 (s, 3H, OAc), 2.01 (s, 3H, OAc), 2.08 (s, 3H, OAc), 2.14 (s, 3H, OAc), 2.23 (t, 1H, J = 6.4 Hz, 0.5 × $CH_2CO_2{}^tBu$), 2.43 (t, 1H, J = 6.4 Hz, 0.5 × $CH_2CO_2{}^tBu$), 3.11−3.69 (m, 6H, 2 × CH_2-N, CH_2-O-Man), 3.95 (m, 1H, $H_{5'}$), 4.07 (dd, 1H, J = 2.1, 12.1 Hz, 0.5 × $H_{6'}$), 4.25 (m, 2H, CH Fmoc, 0.5 × $H_{6'}$), 4.52 (m, 2H, CH_2 Fmoc), 4.75 (m, 1H, $H_{1'}$), 5.25 (m, 3H, $H_{2'}$, $H_{3'}$, $H_{4'}$), 7.30 (t, 2H, J = 7.4 Hz, 2 × CH aromatic), 7.38 (t, 2H, J = 7.4 Hz, 2 × CH aromatic), 7.56 (m, 2H, 2 × CH aromatic), 7.75 (d, 2H, J = 7.4 Hz, 2 × CH aromatic). HRMS (ESI): calculated for $C_{39}H_{50}NO_{14}$ $[M + H]^+$, 756.3231; found, 756.3228.

***β*-Dipeptoid *O*-GalNAc Scaffold 5.** Compound **3** (355 mg, 0.5 mmol) was dissolved in TFA/CH_2Cl_2 (1:1, 6.8 mL) at 0 °C and the reaction was stirred for 1 h at room temperature under an argon atmosphere and then concentrated to dryness. The excess of TFA was removed by coevaporation with CH_2Cl_2 under reduced pressure to give the acid as a yellow foam (328 mg, quantitative), used in the next step without further purification. A stirred solution of the previous acid (328 mg, 0.5 mmol) in anhydrous CH_2Cl_2 (3.0 mL) and DMF (1.8 mL) was treated with DIEA (0.2 mL, 1.2 mmol) for 10 min at room temperature under an argon atmosphere. HATU (358 mg, 0.94 mmol) was added, and the reaction mixture was stirred for an additional 10 min. After that, the monomer amine **1** (251 mg, 0.5 mmol) in anhydrous CH_2Cl_2 (1.0 mL) was added, and the mixture was stirred overnight at the same temperature. After evaporation of the solvent, the crude material was dissolved in CH_2Cl_2 (30 mL), washed with 5% citric acid solution (2 × 30 mL), saturated $NaHCO_3$ solution (2 × 30 mL), H_2O (2 × 30 mL), and brine (2 × 30 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure and the crude was purified by column chromatography (CH_2Cl_2/MeOH, 97:3) to provide compound **5** (480 mg, 81%) as a white foam. RP-HPLC t_R, 16.7 min (77.7%) and 18.1 min (19.9%), $MeOH/H_2O$ 80:20, 97.6% purity, 210 nm. IR (ATR): 3307, 2953, 1745, 1715, 1676, 1648, 1555, 1522, 1479, 1426, 1370, 1223, 1153, 1132, 1047, 1044, 944, 769, 745 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 1.42 (m, 9H, *t*-Bu), 1.49−1.84 (m, 4H, 2 × $CH_2CH_2CH_2$), 1.93−2.14 (m, 24H, 6 × OAc, 2 × NHAc), 2.26−2.71 (m, 4H, 2 × $CH_2C{=}O$), 3.06−3.76 (m, 12H, 4 × CH_2-N, 2 × CH_2-O-GalNAc), 3.98−4.23 (m, 7H, 2 × $H_{5'}$, 4 × $H_{6'}$, CH Fmoc), 4.38−4.63 (m, 4H, 2 × $H_{2'}$, CH_2 Fmoc), 4.84 (m, 2H, 2 × $H_{1'}$), 5.13 (m, 2H, 2 × $H_{3'}$), 5.35 (m, 2H, 2 × $H_{4'}$), 6.34−7.10 (m, 2H, 2 × NHAc), 7.30 (t, 2H, J = 7.4 Hz, 2 × CH aromatic), 7.38 (t, 2H, J = 7.4 Hz, 2 × CH aromatic), 7.54 (m, 2H, 2 × CH aromatic), 7.75 (d, 2H, J = 7.4 Hz, 2 × CH aromatic). HRMS (ESI): calculated for $C_{59}H_{81}N_4O_{23}$ $[M + H]^+$, 1213.5292; found, 1213.5251.

***β*-Dipeptoid O-Mannosylated Scaffold 6.** Compound **4** was conjugated to monomer amine **2** following the procedure described for **5** to yield compound **6** (690 mg, 90%) as a white foam. RP-HPLC t_R, 22.3 min, $MeOH/H_2O$ 80:20, 96.9% purity, 214 nm. IR (ATR): 2947, 1746, 1698, 1643, 1479, 1450, 1440, 1422, 1368, 1220, 1136, 1084, 1047, 980, 743 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 1.43 (m, 9H, *t*-Bu), 1.57−1.83 (m, 4H, 2 × $CH_2CH_2CH_2$), 1.95−2.14 (m, 24H, 8 × OAc), 2.30−2.60 (m, 4H, 2 × $CH_2C{=}O$), 3.10−3.71 (m, 12H, 4 × CH_2-N, 2 × CH_2-O-Man), 3.96 (m, 2H, 2 × $H_{5'}$), 4.1 (m, 2H, 2 × $H_{6'}$), 4.27 (m, 3H, 2 × $H_{6'}$, CH Fmoc), 4.52 (m, 2H, CH_2 Fmoc), 4.77 (m, 2H, 2 × $H_{1'}$), 5.25 (m, 6H, 2 × $H_{2'}$, 2 × $H_{3'}$, 2 × $H_{4'}$), 7.3 (m, 2H, 2 × CH aromatic), 7.38 (t, 2H, J = 7.5 Hz, 2 × CH aromatic), 7.57 (d, 2H, J = 7.5 Hz, 2 × CH aromatic), 7.75 (d, 2H, J = 7.5 Hz, 2 × CH aromatic). HRMS (ESI): calculated for $C_{59}H_{79}N_2O_{25}$ $[M + H]^+$, 1215.4972; found, 1215.4957.

***β*-Tripeptoid *O*-GalNAc Scaffold 7.** Compound **5** was conjugated to monomer amine **1** following the procedure described for **5** to yield compound **7** (380 mg, 75%) as a white foam. RP-HPLC t_R, 14.3 min (70.2%) and 15.4 min (27.5%), $MeOH/H_2O$ 80:20, 97.7% purity, 210 nm. IR (ATR): 3342, 2954, 1747, 1703, 1680, 1634, 1626, 1555, 1531, 1480, 1452, 1435, 1371, 1223, 1155, 1131, 1047, 1044, 949, 734 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 1.42 (m, 9H, *t*-Bu), 1.72−2.13 (m, 42H, 9 × OAc, 3 × NHAc, 3 × $CH_2CH_2CH_2$), 2.18−2.65 (m, 6H, 3 × $CH_2C{=}O$), 3.06−3.69 (m, 18H, 6 × CH_2-N, 3 × CH_2-O-GalNAc), 4.04−4.27 (m, 10H, 3 × $H_{5'}$, 6 × $H_{6'}$, CH Fmoc), 4.47−4.62 (m, 5H, 3 × $H_{2'}$, CH_2 Fmoc), 4.88 (m, 3H, 3 × $H_{1'}$), 5.13 (m, 3H, 3 × $H_{3'}$), 5.36 (m, 3H, 3 × $H_{4'}$), 6.23−7.09 (m, 3H, 3 × NHAc) 7.29 (t, 2H, J = 7.2 Hz, 2 × CH aromatic), 7.38 (t, 2H, J = 7.2 Hz, 2 × CH aromatic), 7.53 (m, 2H, 2 × CH aromatic), 7.74 (d, 2H, J = 7.2 Hz, 2 × CH aromatic). HRMS (ESI): calculated for $C_{79}H_{112}N_6O_{33}$ $[M + H]^{2+}$, 836.3630; found, 836.3607.

***β*-Tripeptoid O-Mannosylated Scaffold 8.** Compound **6** was conjugated to monomer amine **2** following the procedure described for **5** to yield compound **8** (738 mg, 87%) as a white foam. RP-HPLC t_R, 21.4 min, $MeOH/H_2O$ 80:20, 96.6% purity, 214 nm. IR (ATR): 2937, 1749, 1699, 1645, 1640, 1480, 1458, 1441, 1423, 1370, 1223, 1136, 1084, 1047, 970 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 1.4 (m, 9H, *t*-Bu), 1.60−1.80 (m, 6H, 3 × $CH_2CH_2CH_2$), 1.91−2.15 (m, 36H, 12 × OAc), 2.40−2.62 (m, 6H, 3 × $CH_2C{=}O$), 3.10−3.70 (m,

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18H, 6 × CH_2-N, 3 × CH_2-O-Man), 3.94 (m, 3H, 3 × H$_{5'}$), 4.06 (m, 3H, 3 × H$_{6'}$), 4.25 (m, 4H, 3 × H$_{6'}$, CH Fmoc), 4.47 (m, 2H, CH_2 Fmoc), 4.75 (m, 3H, 3 × H$_{1'}$), 5.25 (m, 9H, 3 × H$_{2'}$, 3 × H$_{3'}$, 3 × H$_{4'}$), 7.29 (m, 2H, 2 × CH aromatic), 7.36 (t, 2H, J = 7.5 Hz, 2 × CH aromatic), 7.56 (d, 2H, J = 7.5 Hz, 2 × CH aromatic), 7.73 (d, 2H, J = 7.5 Hz, 2 × CH aromatic). HRMS (ESI): calculated for $C_{79}H_{109}N_3O_{36}$ $[M + H]^{2+}$, 837.8395; found, 837.8367.

β-Tripeptoid *O*-GalNAc Scaffold 9. A stirred solution of compound **7** (50 mg, 0.03 mmol) in CH_3CN (0.8 mL) was treated with Et_2NH (0.4 mL) at 0 °C for 30 min and at room temperature for 4 h. After concentration to dryness, the excess of Et_2NH was removed by coevaporation with CH_2Cl_2 and the crude was purified by column chromatography (CH_2Cl_2/MeOH, 95:5 to 85:15) to obtain compound **9** (46 mg, quantitative) as a yellow oil. IR (ATR): 3322, 2942, 1745, 1732, 1654, 1636, 1542, 1536, 1432, 1426, 1371, 1222, 1225, 1156, 1130, 1048, 1036 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 1.39−1.42 (m, 9H, m, *t*-Bu), 1.70−2.13 (m, 43H, 9 × OAc, 3 × NHAc, 3 × $CH_2CH_2CH_2$, N*H* peptoid), 2.18−2.83 (m, 6H, 3 × CH_2C═O), 2.90−4.01 (m, 18H, 6 × CH_2-N, 3 × CH_2-O-GalNAc), 4.03−4.36 (m, 9H, 3 × H$_{5'}$, 6 × H$_{6'}$), 4.58 (m, 3H, 3 × H$_{2'}$), 4.82−5.00 (m, 3H, 3 × H$_{1'}$), 5.13 (m, 3H, 3 × H$_{3'}$), 5.36 (m, 3H, 3 × H$_{4'}$), 6.54−7.45 (m, 3H, 3 × N*H*Ac). HRMS (ESI): calculated for $C_{64}H_{102}N_6O_{31}$ $[M + H]^{2+}$, 725.3295; found, 725.3262.

β-Tripeptoid O-Mannosylated Scaffold 10. A procedure similar to that for compound **9** was used. The product **10** (33 mg, quantitative) was obtained as a yellow oil from **8**. IR (ATR): 2949, 2916, 1745, 1741, 1641, 1452, 1440, 1431, 1371, 1224, 1137, 1082, 1045 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 1.42 (m, 9H, *t*-Bu), 1.78−2.14 (m, 42H, 12 × OAc, 3 × $CH_2CH_2CH_2$), 2.24 (m, 1H, N*H* peptoid), 2.47−2.79 (m, 4H, 2 × CH_2C═O), 2.93−3.86 (m, 20H, 0.5 × CH_2C═O, 6 × CH_2-N, 3 × CH_2-O-Man), 3.96 (m, 3H, 3 × H$_{5'}$), 4.07 (m, 3H, 3 × H$_{6'}$), 4.27 (dd, 3H, J = 5.2, 12.0 Hz, 3 × H$_{6'}$), 4.77−4.84 (m, 3H, 3 × H$_{1'}$), 5.17−5.34 (m, 9H, 3 × H$_{2'}$, 3 × H$_{3'}$, 3 × H$_{4'}$). HRMS (ESI): calculated for $C_{64}H_{99}N_3O_{34}$ $[M + H]^{2+}$, 726.8055; found, 726.8067.

β-Tripeptoid *O*-GalNAc Scaffold 11. A stirred solution of compound **9** (7.10 mg, 0.0049 mmol) in dry MeOH (1.3 mL) was treated with a solution of NaOMe (130 μL) in dry MeOH for 1 h at room temperature under an argon atmosphere. The reaction mixture was neutralized with Dowex 50W-X8 (H+) resin and then filtered to give the compound **11** (5.2 mg, quantitative) as a white solid. IR (ATR): 3514, 3160, 2926, 2853, 1710, 1653, 1634, 1623, 1554, 1467, 1342, 1233, 1154, 1123, 1058, 1041 cm^{-1}. ^1H NMR (400 MHz, CD_3OD) δ 1.42 (m, 9H, *t*-Bu), 1.80−2.09 (m, 15H, 3 × $CH_2CH_2CH_2$, 3 × NHAc), 2.50−3.00 (m, 6H, 3 × CH_2C═O), 3.20−3.90 (m, 33H, 3 × CH_2CH_2C═O, 3 × CH_2N, 3 × OCH_2CH_2, 3 × H$_{3'}$, 3 × H$_{4'}$, 3 × H$_{5'}$, 6 × H$_{6'}$), 4.30 (m, 3H, 3H$_{2'}$), 4.70 (m, 3H, 3H$_{1'}$). HRMS (ESI): calculated for $C_{46}H_{83}N_6O_{22}$ $[M + H]^+$, 536.2819; found, 536.2825.

β-Dipeptoid O-Mannosylated Scaffold 12. A procedure similar to that for compound **11** was used. The product **12** was obtained (8.0 mg, quantitative) as a white foam from **10**. IR (ATR): 3517, 3168, 2926, 2857, 1715, 1656, 1630, 1625, 1555, 1475, 1345, 1233, 1154, 1123, 1060, 1047 cm^{-1}. ^1H NMR (400 MHz, CD_3OD) δ 1.42 (m, 9H, *t*-Bu), 1.80−2.09 (m, 6H, 3 × $CH_2CH_2CH_2$), 2.50−3.00 (m, 6H, 3 × CH_2C═O), 3.20−3.90 (m, 36H, 3 × CH_2CH_2C═O, 3 × CH_2N, 3 × OCH_2CH_2, 3 × H$_{2'}$, 3 × H$_{3'}$, 3 × H$_{4'}$, 3 × H$_{5'}$, 6 × H$_{6'}$), 4.70 (m, 3H, 3 × H$_{1'}$). HRMS (ESI): calculated for $C_{40}H_{74}N_3O_{22}$ $[M + H]^+$, 948.4764; found, 948.4776.

β-Tripeptoid *O*-GalNAc Scaffold 13. A procedure similar to that for compound **5** was used. The reaction was carried out with compound **7** (1.0 equiv), conjugated to propargyl amine (3.0 equiv) to yield compound **13** (138 mg, 95%) as a white foam. RP-HPLC t_R, 10.0 min (75.8%) and 10.5 min (24.1%), MeOH/H_2O 80:20, 99.9% purity, 210 nm. IR (ATR): 3289, 2948, 2371, 1745, 1675, 1671, 1648, 1637, 1570, 1541, 1478, 1456, 1424, 1371, 1224, 1166, 1131, 1073, 1047, 948, 923, 742 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 1.70−2.21 (m, 43H, 9 × OAc, 3 × NHAc, C≡C*H*, 3 × $CH_2CH_2CH_2$), 2.24−2.68 (m, 6H, 3 × CH_2C═O), 3.11−3.72 (m, 18H, 6 × CH_2-N, 3 × CH_2-O-GalNAc), 3.96−4.26 (m, 12H, CH_2-NH, 3 × H$_{5'}$, 6 × H$_{6'}$, CH Fmoc), 4.56 (m, 5H, 3 × H$_{2'}$, CH_2 Fmoc), 4.86 (m, 3H, 3 × H$_{1'}$), 5.14 (m, 3H, 3 × H$_{3'}$), 5.35 (m, 3H, 3 × H$_{4'}$), 6.54−7.18 (m, 4H, 3 × N*H*Ac, N*H*-CH_2C≡CH), 7.27 (m, 2H, 2 × CH aromatic), 7.37 (m, 2H, 2 × CH aromatic), 7.53 (m, 2H, 2 × CH aromatic), 7.73 (m, 2H, 2 × CH aromatic). HRMS (ESI): calculated for $C_{78}H_{107}N_7O_{32}$ $[M + H]^{2+}$, 826.8475; found, 826.8450.

β-Tripeptoid O-Mannosylated Scaffold 14. A procedure similar to that for compound **5** was used. The reaction was carried out with compound **8** (1.0 equiv), conjugated to propargyl amine (3.0 equiv) to yield compound **14** (278 mg, 86%) as a white foam. RP-HPLC t_R, 12.8 min, MeOH/H_2O 80:20, 98.0% purity, 214 nm. IR (ATR): 2941, 2360, 1744, 1693, 1672, 1635, 1477, 1456, 1445, 1426, 1369, 1219, 1135, 1083, 1047, 979, 747 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 1.56−1.82 (m, 6H, 3 × $CH_2CH_2CH_2$), 1.94−2.12 (m, 36H, 12 × OAc), 2.20 (m, 1H, C≡C*H*), 2.45−2.7 (m, 6H, 3 × CH_2C═O), 3.15−3.7 (m, 18H, 6 × CH_2-N, 3 × CH_2-O-Man), 3.94 (m, 5H, CH_2-NH, 3 × H$_{5'}$), 4.07 (m, 3H, 3 × H$_{6'}$), 4.25 (m, 4H, 3 × H$_{6'}$, CH Fmoc), 4.47 (m, 2H, CH_2 Fmoc), 4.75 (m, 3H, 3 × H$_{1'}$), 5.22 (m, 9H, 3 × H$_{2'}$, 3 × H$_{3'}$, 3 × H$_{4'}$), 7.22 (m, 1H, N*H*-CH_2C≡CH), 7.29 (m, 2H, 2 × CH aromatic), 7.36 (t, 2H, J = 7.1 Hz, 2 × CH aromatic), 7.56 (d, 2H, J = 7.1 Hz, 2 × CH aromatic), 7.73 (d, 2H, J = 7.1 Hz, 2 × CH aromatic). HRMS (ESI): calculated for $C_{78}H_{104}N_4O_{35}$ $[M + H]^{2+}$, 828.3241; found, 828.3207.

β-Tripeptoid *O*-GalNAc Scaffold 15. A procedure similar to that for compound **9** was used. The product **15** (53 mg, quantitative) was obtained as a yellow oil from **13**. IR (ATR): 3262, 2914, 2378, 1746, 1673, 1654, 1648, 1637, 1577, 1539, 1528, 1451, 1430, 1372, 1220, 1223, 1167, 1170, 1133, 1142, 1048, 1037 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 1.73−2.92 (m, 45H, 9 × OAc, 3 × NHAc, C≡C*H*, 3 × CH_2C═O, 3 × $CH_2CH_2CH_2$, N*H* peptoid), 3.10−4.05 (20H, m, NH−CH_2-C≡CH, 6 × CH_2-N, 3 × CH_2-O-GalNAc), 4.07−4.47 (m, 9H, 3 × H$_{5'}$, 6 × H$_{6'}$), 4.59 (m, 3H, 3 × H$_{2'}$), 4.92 (m, 3H, 3 × H$_{1'}$), 5.12 (m, 3H, 3 × H$_{3'}$), 5.36 (m, 3H, 3 × H$_{4'}$), 6.67−8.00 (m, 4H, 3 × N*H*Ac, N*H*-CH_2C≡CH). HRMS (ESI): calculated for $C_{63}H_{97}N_7O_{30}$ $[M + H]^{2+}$, 715.8135; found, 715.8030.

β-Tripeptoid O-Mannosylated Scaffold 16. A procedure similar to that for compound **9** was used. Product **16** (51 mg, quantitative) was obtained as a yellow oil from **14**. IR (ATR): 2954, 2360, 1744, 1674, 1668, 1550, 1452, 1432, 1370, 1223, 1135, 1084, 1047 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 1.81 (m, 6H, 3 × $CH_2CH_2CH_2$), 1.94−2.11 (m, 36H, 12 × OAc), 2.20 (m, 2H, C≡C*H*, N*H* peptoid), 2.49−3.02 (m, 6H, 3 × CH_2C═O), 3.08−3.77 (m, 18H, 6 × CH_2-N, 3 × CH_2-O-Man), 3.95 (m, 5H, NH−CH_2-C≡CH, 3 × H$_{5'}$), 4.07 (m, 3H, 6 × H$_{6'}$), 4.25 (m, 3H, 6 × H$_{6'}$), 4.80 (m, 3H, 3 × H$_{1'}$), 5.22 (m, 9H, 3 × H$_{2'}$, 3 × H$_{3'}$, 3 × H$_{4'}$), 7.75 (m, 1H, N*H*-CH_2C≡CH). HRMS (ESI): calculated for $C_{63}H_{94}N_4O_{33}$ $[M + H]^{2+}$, 717.2900; found, 717.2973.

TLR7 Agonist−Spacer Conjugate 26. A stirred solution of agonist TLR7 **24** (70 mg, 0.2 mmol) in dry DMF (4 mL) was treated with tributylamine (70 μL, 0.3 mmol) for 10 min at room temperature under an argon atmosphere, and then isobutyl chloroformate (38 μL, 0.3 mmol) was added. After 10 min again, aminocaproic acid (128 mg, 0.98 mmol) in DMF/H_2O (1:1, 4 mL) was added at 0 °C and the mixture was stirred for an additional 28 h. The reaction mixture was concentrated, dissolved in H_2O (7 mL), and acidified with 1.0 M HCl to pH 1.0. The mixture was cooled down to 0 °C and then filtered to give compound **26** (51 mg, 55%) as a white solid. IR (ATR): 3419, 3103, 3039, 2935, 2858, 1705, 1636, 1616, 1553, 1506, 1452, 1419, 1375, 1348, 1327, 1125, 1092, 949, 779, 717 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 1.26 (m, 2H, $CH_2(CH_2)_2CO_2H$), 1.50 (m, 4H, $NHCH_2CH_2$, $CH_2CH_2CO_2H$), 2.19 (t, 2H, J = 7.2 Hz, CH_2CO_2H), 3.21 (m, 2H, $NHCH_2$), 3.26 (s, 3H, CH_3-O), 3.56 (t, 2H, J = 4.5 Hz, CH_2-O), 4.24 (t, 2H, J = 4.5 Hz, CH_2-O), 4.89 (s, 2H, CH_2-C_6H_4C═O), 6.53 (s, 2H, NH_2), 7.34 (d, 2H, J = 8.0 Hz, 2 × CH aromatic), 7.76 (d, 2H, J = 8.0 Hz, 2 × CH aromatic), 8.39 (t, 1H, J = 5.0 Hz, N*H*C═O), 10.0 (s, 1H, N*H* cycle), 12.0 (s, 1H, CO_2H). HRMS (ESI): calculated for $C_{22}H_{29}N_6O_6$ $[M + H]^+$, 473.2149; found, 473.2146.

TLR7 Agonist−Spacer−*O*-GalNAc β-Tripeptoid Conjugate 27. A stirred solution of the acid **26** (10.4 mg, 0.022 mmol) in

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anhydrous CH_2Cl_2 (0.5 mL) and DMF (0.45 mL) was treated with DIEA (10 μL, 0.051 mmol) for 10 min at room temperature under an argon atmosphere. HATU (16.7 mg, 0.044 mmol) was added, and the reaction mixture was stirred for an additional 10 min. After that, the monomer amine **9** (31.9 mg, 0.022 mmol) in anhydrous CH_2Cl_2 (0.55 mL) and the mixture were stirred for 40 h at the same temperature. After evaporation of the solvent, the crude material was dissolved in CH_2Cl_2 (15 mL), washed with 5% citric acid solution (2 × 15 mL), saturated $NaHCO_3$ solution (2 × 15 mL), H_2O (2 × 15 mL), and brine (2 × 15 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure and the crude was purified by column chromatography (AcOEt/MeOH, 90:10 to 80:20) to provide compound **27** (22 mg, 53%) as a white foam. IR (ATR): 3354, 3330, 2960, 2923, 2853, 1746, 1733, 1656, 1649, 1640, 1633, 1622, 1615, 1570, 1541, 1460, 1436, 1428, 1371, 1259, 1233, 1227, 1158, 1153, 1129, 1081, 1075, 1035, 800 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 1.43 (s, 9H, *t*-Bu), 1.54−1.85 (m, 12H, 3 × $CH_2CH_2CH_2$, $CH_2CH_2C{=}O$ spacer, $CH_2CH_2CH_2C{=}O$ spacer, CH_2CH_2NH spacer), 1.90−2.16 (m, 36H, 9 × OAc, 3 × NHAc), 2.20−2.70 (m, 8H, 3 × $CH_2CO_2{}^tBu$, $CH_2C{=}O$ spacer), 3.30−3.74 (m, 25H, 6 × CH_2N peptoid, 3 × CH_2-O-GalNAc, CH_2NH spacer, CH_2-O, CH_3-O), 4.05−4.27 (m, 9H, 3 × $H_{5'}$, 6 × $H_{6'}$), 4.43 (m, 2H, CH_2-O), 4.53−4.64 (m, 3H, 3 × $H_{2'}$), 4.85−5.08 (m, 5H, 3 × $H_{1'}$, CH_2N agonist), 5.15 (m, 3H, 3 × $H_{3'}$), 5.37 (m, 3H, 3 × $H_{4'}$), 5.77 (m, 2H, NH_2), 6.76−7.73 (m, 8H, 4 × C*H* aromatic, N*H* spacer, 3 × N*H*Ac), 10.16 (m, 1H, NH cycle). HRMS (ESI): calculated for $C_{86}H_{128}N_{12}O_{36}$ $[M + H]^{2+}$, 952.4277; found, 952.4245.

TLR7 Agonist−Spacer−O-Mannosylated β-Tripeptoid Conjugate 28. A procedure similar to that for compound **27** was used. Compound **26** was conjugated to the glycopeptoid **10** to yield compound **28** (23 mg, 53%) as a white foam. IR (ATR): 3352, 3329, 2962, 2925, 2858, 1745, 1733, 1657, 1645, 1640, 1633, 1628, 1617, 1571, 1541, 1460, 1438, 1428, 1378, 1260, 1233, 1222, 1159, 1153, 1130, 1081, 1076, 1034, 800 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 1.42 (s, 1H, *t*-Bu), 1.58−1.90 (m, 12H, 3 × $CH_2CH_2CH_2$, $CH_2CH_2C{=}O$ spacer, $CH_2CH_2CH_2C{=}O$ spacer, CH_2CH_2NH spacer), 1.93−2.15 (m, 36H, 12 × OAc), 2.25−2.72 (m, 8H, 3 × $CH_2CO_2{}^tBu$, $CH_2C{=}O$ spacer), 3.34−3.76 (m, 25H, 6 × CH_2N peptoid, 3 × CH_2-O-Man, CH_2N spacer, CH_2-O, CH_3-O), 3.97 (m, 3H, 3 × $H_{5'}$), 4.09 (m, 3H, 3 × $H_{6'}$), 4.28 (dd, 3H, *J* = 4.7, 12.1 Hz, 3 × $H_{6'}$), 4.41 (m, 2H, CH_2-O), 4.81 (m, 3H, 3 × $H_{1'}$), 5.00 (m, 2H, CH_2N agonist), 5.16−5.31 (m, 9H, 3 × $H_{2'}$, 3 × $H_{3'}$, 3 × $H_{4'}$), 5.73 (m, 2H, NH_2), 7.36−7.44 (m, 3H, 2 × CH aromatic, NH spacer), 7.80 (d, 2H, *J* = 7.9 Hz, 2 × CH aromatic), 10.07 (m, 1H, N*H* cycle). HRMS (ESI): calculated for $C_{86}H_{125}N_9O_{39}$ $[M + H]^{2+}$, 953.9037; found, 953.9047.

TLR7 Agonist−Spacer−*O*-GalNAc β-Tripeptoid Conjugate 29. A procedure similar to that for compound **27** was used. Compound **26** was conjugated to the glycopeptoid **15** to yield compound **29** (20 mg, 48%) as a white foam. RP-HPLC t_R, 9.0 min, MeOH/H_2O 70:30, 98.4% purity, 254 nm. IR (ATR): 3306, 3230, 2960, 2923, 2853, 2354, 1745, 1731, 1648, 1639, 1615, 1571, 1546, 1466, 1426, 1415, 1371, 1343, 1229, 1162, 1130, 1129, 1075, 1045, 781 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 1.39−1.88 (m, 12H, 3 × $CH_2CH_2CH_2$, $CH_2CH_2C{=}O$ spacer, $CH_2CH_2CH_2C{=}O$ spacer, CH_2CH_2NH spacer), 1.94−2.16 (m, 39H, 9 × OAc, 3 × NHAc, C≡*C*H, $CH_2C{=}O$ spacer), 2.3−2.68 (m, 6H, 3 × $CH_2C{=}O$ peptoid), 3.30−3.74 (m, 25H, 6 × CH_2N peptoid, 3 × CH_2-O-GalNAc, CH_2NH spacer, CH_2-O agonist, CH_3-O), 3.94−4.19 (m, 11H, NH−CH_2-C≡CH, 3 × $H_{5'}$, 6 × $H_{6'}$), 4.43 (m, 2H, CH_2-O agonist), 4.58 (m, 3H, 3 × $H_{2'}$), 4.83−5.04 (m, 5H, 3 × $H_{1'}$, CH_2N agonist), 5.09−5.22 (m, 3H, 3 × $H_{3'}$), 5.38 (m, 3H, 3 × $H_{4'}$), 5.76 (m, 2H, NH_2), 6.86−7.72 (m, 9H, 4 × CH aromatic, N*H* spacer, 3 × N*H*Ac, N*H*-CH_2C≡H), 10.16 (m, 1H, N*H* cycle). HRMS (ESI): calculated for $C_{85}H_{123}N_{13}O_{35}$ $[M + H]^{2+}$, 942.9122; found, 942.9157.

TLR7 Agonist−Spacer−O-Mannosylated β-Tripeptoid Conjugate 30. A procedure similar to that for compound **27** was used. Compound **26** was conjugated to the glycopeptoid **16** to yield compound **30** (22 mg, 50%) as a white foam. RP-HPLC t_R, 11.7 min, MeOH/H_2O 70:30, 92.6% purity, 210 nm. IR (ATR): 3355, 3333, 2957, 2924, 1746, 1647, 1644, 1618, 1575, 1540, 1458, 1440, 1419, 1370, 1342, 1225, 1134, 1090, 1047, 932, 899 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 1.58−1.88 (m, 12H, 3 × $CH_2CH_2CH_2$, $CH_2CH_2C{=}O$ spacer, $CH_2CH_2CH_2C{=}O$ spacer, CH_2CH_2NH spacer), 1.95−2.15 (m, 39H, 12 × OAc, C≡*C*H, $CH_2C{=}O$ spacer), 2.19−2.71 (m, 6H, 3 × $CH_2C{=}O$ peptoid), 3.27−3.76 (m, 25H, 6 × CH_2N peptoid, 3 × CH_2-O-Man, CH_2NH spacer, CH_2-O agonist, CH_3-O), 3.9−4.01 (m, 5H, NH−CH_2-C≡CH, 3 × $H_{5'}$), 4.1 (m, 3H, 3 × $H_{6'}$), 4.28 (dd, 3H, *J* = 4.8, 12.2 Hz, 3 × $H_{6'}$), 4.41 (m, 2H, CH_2-O agonist), 4.81 (m, 3H, 3 × $H_{1'}$), 5.00 (m, 2H, CH_2N agonist), 5.17−5.32 (m, 9H, 3 × $H_{2'}$, 3 × $H_{3'}$, 3 × $H_{4'}$), 5.82 (m, 2H, NH_2), 7.39−7.88 (m, 6H, 4 × CH aromatic, N*H* spacer, N*H*-CH_2C≡H), 10.1 (m, 1H, N*H* cycle). HRMS (ESI): calculated for $C_{85}H_{120}N_{10}O_{38}$ $[M + H]^{2+}$, 944.3882; found, 944.3833.

TLR7 Agonist−Spacer−*O*-GalNAc β-Tripeptoid Conjugate 31. A procedure similar to that for compound **11** was used. The product **31** was obtained (12.6 mg, quantitative) as a white foam from **27**. RP-HPLC t_R, 7.4 min, MeOH/H_2O 60:40, 95.9% purity, 214 nm. IR (ATR): 3501, 3181, 2928, 2853, 1720, 1642, 1625, 1615, 1555, 1465, 1374, 1340, 1153, 1123, 1060, 1039, 971 cm^{-1}. ^1H NMR (400 MHz, CD_3OD) δ 1.42−1.45 (m, 9H, *t*-Bu), 1.55−1.71 (m, 6H, m, $CH_2CH_2C{=}O$ spacer, $CH_2CH_2CH_2C{=}O$ spacer, CH_2CH_2NH spacer), 1.75−1.93 (m, 6H, 3 × $CH_2CH_2CH_2$ peptoid), 1.99−2.03 (m, 9H, 3 × NHAc), 2.38−2.88 (m, 8H, 3 × $CH_2CO_2{}^tBu$, $CH_2C{=}O$ spacer), 3.33−3.90 (m, 40H, 6 × CH_2N peptoid, 3 × CH_2-O-GalNAc, 3 × $H_{3'}$, 3 × $H_{4'}$, 3 × $H_{5'}$, 6 × $H_{6'}$, CH_2NH spacer, CH_2-O, CH_3-O), 4.28 (m, 3H, 3 × $H_{2'}$), 4.45 (m, 2H, CH_2-O), 4.75−4.83 (m, 3H, 3 × $H_{1'}$), 5.01 (s, 2H, CH_2N agonist), 7.47 (d, 2H, *J* = 8.2 Hz, 2 × CH aromatic), 7.78 (d, 2H, *J* = 8.2 Hz, 2 × CH aromatic). HRMS (ESI): calculated for $C_{68}H_{110}N_{12}O_{27}$ $[M + H]^{2+}$, 763.3802; found, 763.3774.

TLR7 Agonist−Spacer−O-Mannosylated β-Tripeptoid Conjugate 32. A procedure similar to that for compound **11** was used. The product **32** was obtained (3.9 mg, quantitative) as a white foam from **28**. IR (ATR): 3508, 3175, 2928, 2859, 1710, 1645, 1630, 1620, 1560, 1465, 1373, 1345, 1155, 1120, 1061, 1040, 971 cm^{-1}. ^1H NMR (400 MHz, CD_3OD) δ 1.42−1.45 (m, 9H, *t*-Bu), 1.57−1.71 (m, 6H, $CH_2CH_2C{=}O$ spacer, $CH_2CH_2CH_2C{=}O$ spacer, CH_2CH_2NH spacer), 1.78−1.95 (m, 6H, 3 × $CH_2CH_2CH_2$ peptoid), 2.32−2.84 (m, 8H, 3 × $CH_2CO_2{}^tBu$, $CH_2C{=}O$ spacer), 3.39−3.98 (m, 43H, 6 × CH_2N peptoid, 3 × CH_2-O-Man, 3 × $H_{2'}$, 3 × $H_{3'}$, 3 × $H_{4'}$, 3 × $H_{5'}$, 6 × $H_{6'}$, CH_2NH spacer, CH_2-O, CH_3-O), 4.45 (m, 2H, CH_2-O), 4.72 (m, 3H, 3 × $H_{1'}$), 5.07 (s, 2H, CH_2N agonist), 7.50 (d, 2H, *J* = 8.1 Hz, 2 × CH aromatic), 7.80 (d, 2H, *J* = 7.7 Hz, 2 × CH aromatic). HRMS (ESI): calculated for $C_{62}H_{101}N_9O_{27}$ $[M + H]^{2+}$, 701.8403; found, 701.8415.

TLR7 Agonist−Spacer−*O*-GalNAc β-Tripeptoid Conjugate 33. A procedure similar to that for compound **11** was used. The product **33** was obtained (12.1 mg, quantitative) as a white foam from **29**. RP-HPLC t_R, 11.0 min, 10−60% MeOH/H_2O, 85.6% purity, 205 nm. IR (ATR): 3342, 3264, 2950, 2925, 2853, 2364, 1718, 1710, 1648, 1637, 1618, 1560, 1545, 1460, 1450, 1439, 1419, 1377, 1342, 1154, 1121, 1039, 787 cm^{-1}. ^1H NMR (400 MHz, CD_3OD) δ 1,58−1,71 (m, 6H, $CH_2CH_2C{=}O$ spacer, $CH_2CH_2CH_2C{=}O$ spacer, CH_2CH_2NH spacer), 1.78−1.93 (m, 6H, 3 × $CH_2CH_2CH_2$), 1.99−2,05 (m, 9H, 3 × NHAc), 2.39−2.85 (m, 9H, C≡*C*H, 3× $CH_2C{=}O$ peptoid, $CH_2C{=}O$ spacer), 3.36−3.95 (m, 42H, NH−CH_2-C≡CH, 6 × CH_2N peptoid, 3 × CH_2-O-GalNAc, 3 × $H_{3'}$, 3 × $H_{4'}$, 3 × $H_{5'}$, 3 × $H_{5'}$, CH_2NH spacer, CH_2-O agonist, O−CH_3), 4.28 (m, 3H, 3 × $H_{2'}$), 4.40 (m, 2H, CH_2-O agonist), 4.76−4.84 (m, 3H, 3 × $H_{1'}$), 5.04 (s, 2H, CH_2N agonist), 7.47 (d, 2H, *J* = 8.2 Hz, 2 × CH aromatic), 7.78 (d, 2H, *J* = 8.2 Hz, 2 × CH aromatic). HRMS (ESI): calculated for $C_{67}H_{105}N_{13}O_{26}$ $[M + H]^{2+}$, 753.8642; found, 753.8641.

TLR7 Agonist−Spacer−O-Mannosylated β-Tripeptoid Conjugate 34. A procedure similar to that for compound **11** was used. The product **34** was obtained (10.8 mg, quantitative) as a white foam from **30**. RP-HPLC t_R, 10.6 min, 10−60% MeOH/H_2O, 89.8% purity, 210 nm. IR (ATR): 3532, 3120, 2924, 2855, 2359, 1727, 1707, 1663, 1617, 1564, 1465, 1419, 1343, 1200, 1132, 1062, 1030, 1028, 972 cm^{-1}. ^1H NMR (400 MHz, CD_3OD) δ 1.57−1.71 (m, 6H,

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$CH_2CH_2C{=}O$ spacer, $CH_2CH_2CH_2C{=}O$ spacer, CH_2CH_2NH spacer), 1.77−1.92 (m, 6H, 3 × $CH_2CH_2CH_2$), 2.35−2.84 (m, 9H, $C{\equiv}CH$, 3× $CH_2C{=}O$ peptoid, $CH_2C{=}O$ spacer), 3.36−3.97 (m, 45H, NH−CH_2-C≡CH, 6 × CH_2N peptoid, 3 × CH_2-O-Man, 3 × $H_{2'}$, 3 × $H_{3'}$, 3 × $H_{4'}$, 3 × $H_{5'}$, 3 × $H_{5''}$, CH_2NH spacer, CH_2-O agonist, O−CH_3), 4.41 (m, 2H, CH_2-O agonist), 4.74 (3H, m, 3 × $H_{1'}$), 5.04 (m, 2H, CH_2N agonist), 7.46 (d, 2H, J = 8.1 Hz, 2 × CH aromatic), 7.78 (d, 2H, J = 7.7 Hz, 2 × CH aromatic). HRMS (ESI): calculated for $C_{61}H_{96}N_{10}O_{26}$ $[M + H]^{2+}$, 692.3243; found, 692.3209.

Three-Component *O*-GalNAc Construct 35. All solvents were degassed by several vacuum (15 mbar)/argon cycles. A stirred solution of compound **33** (3.9 mg, 0.0026 mmol) and OVA 323−339 peptide (5.0 mg, 0.0026 mmol) was dissolved in HFIP (150 μL), H_2O (50 μL), and HEPES buffer (100 μL, 100 mM, pH 7.5) at room temperature under an argon atmosphere. This solution was neutralized to pH 7.5 with 0.5 N NaOH. Then, a catalytic solution (350 μL) composed of $CuSO_4{\cdot}5H_2O$ (2.6 mg, 0.01 mmol), THPTA (6.8 mg, 0.016 mmol), aminoguanidine hydrochloride (2.9 mg, 0.026 mmol), and sodium ascorbate (4.2 mg, 0.02 mmol) was added. The reaction mixture was stirred for 1 h, quenched with 0.5% aqueous TFA, and purified on SPE cartridge (H_2O/CH_3CN, 100:0 to 0:100) to afford the three-component *O*-GalNAc **35** (7.1 mg, 80%) as a white solid after lyophilization. RP-HPLC t_R, 7.1 min, CH_3CN/H_2O (0.1% TFA) 30:70, 96.5% purity, 214 nm. HRMS (ESI): calculated for $C_{147}H_{235}N_{42}O_{52}$ $[M + H]^{3+}$, 1140.2340; found, 1140.5516; for $C_{147}H_{236}N_{42}O_{52}$ $[M + H]^{4+}$, 855.4273; found, 855.6634. MALDI-TOF: calculated for $[M + H]^+$ 3418.6874; found,3418.6375.

Three-Component O-Mannosylated Construct 36. All solvent were degassed by several vacuum (15 mbar)/argon cycles. A stirred solution of compound **34** (3.6 mg, 0.0026 mmol) and OVA 323−339 peptide (5.0 mg, 0.0026 mmol) was dissolved in HFIP (150 μL), H_2O (50 μL) and HEPES buffer (100 μL, 100 mM, pH 7.5) at room temperature under an argon atmosphere. This solution was neutralized to pH 7.5 with 0.5 N NaOH. Then, a catalytic solution (350 μL) composed of $CuSO_4{\cdot}5H_2O$ (1.3 mg, 0.013 mmol), THPTA (6.8 mg, 0.016 mmol), aminoguanidine hydrochloride (1.4 mg, 0.013 mmol), and sodium ascorbate (2.1 mg, 0.01 mmol) was added. Under these conditions, **34** and OVA were not totally consumed after 1 h. Another catalytic solution was added with the same amount of each compound except for $CuSO_4{\cdot}5H_2O$ (2.6 mg, 0.026 mmol) and sodium ascorbate (4.2 mg, 0.02 mmol). After 2 h of reaction, the same amounts of $CuSO_4{\cdot}5H_2O$ and sodium ascorbate were added again. Finally, a complete conversion was observed within 3 h and the reaction mixture was quenched with 0.5% aqueous TFA and purified on SPE cartridge (H_2O/CH_3CN, 100:0 to 0:100) to afford the three-component O-mannosylated **36** (6.6 mg, 80%) as a white solid after lyophilization. RP-HPLC t_R, 7.2 min, CH_3CN/H_2O (0.1% TFA) 30:70, 95.8% purity, 214 nm. HRMS (ESI): calculated for $C_{141}H_{226}N_{39}O_{52}$ $[M + H]^{3+}$, 1099.2074; found, 1099.5420; for $C_{141}H_{227}N_{39}O_{52}$ $[M + H]^{4+}$, 824.6574; found, 824.8961. MALDI-TOF: calculated for $[M + H]^+$, 3295.6077; found, 3295,6024.

Immunology: Experimental Part. Stimulation of Mouse Bone Marrow-Derived DCs (BMDCs) by the Compounds (Figure 2A and Figure 2B). Bone marrow-derived DCs were generated from bone marrow precursors from C57BL/6 mice in RPMI medium (Gibco) containing 50 mM 2-ME (Gibco), 10% fetal calf serum (Thermo Hyclone), antibiotics, and 1% of a GM-CSF-containing supernatant. Cells were recovered on day 6 with PBS EDTA and usually contained 60−70% of $CD11c^+CD11b^+$ cells. They were then incubated at 37 °C with increasing concentrations of the various compounds (**11**, **24**, **31**, **35**, and **36**) for 48 h. IL-12p40 and IL-6 were measured in cell supernatants by ELISA.

IFN-γ Production by OVA-Specific OT-II T Cells in Response to the Constructs (Figure 2C). C57BL/6 bone marrow-derived DC were incubated in RPMI medium (Gibco) containing 50 mM 2-ME (Gibco), 10% fetal calf serum (Thermo Hyclone), and antibiotics at 37 °C with increasing concentrations of the compound **31**, **35**, or **36**. $CD4^+$ T lymphocytes isolated from OT-II Rag2 T cell receptor-transgenic knockout mice, bred at the animal facilities of the Institut Pasteur, and specific for the I-A^b-$OVA_{323-339}$ epitopes were added to the cultures for 3 days. IFN-γ was then measured in cell supernatants by ELISA.

In Vivo T-Immunogenicity (Figure 3). C57BL/6 mice from Charles River Laboratories (Les Oncins, France) were used at 6−10 weeks of age. OT-II cells were harvested from OT-II Rag2 T cell receptor-transgenic knockout mice, bred at the animal facilities of the Pasteur Institute. OT-2 cells are specific for the $OVA_{323-339}$ T-cell epitope presented by I-A^b molecules. After administration of 2 × 10^5 OT-II $CD4^+$ T cells, C57BL/6 mice (n = 3 per group) were intradermally immunized with **24**, **31**, **35**, **36**, or OVA 323−339 compounds (11.7 nmol per mouse).

OT-II $CD4^+$ T Cells Proliferation (Figure 3A). Five days after immunization, C57BL/6 mice were sacrificed and the spleen was collected. The in vivo proliferation of OT-II $CD4^+$ T cells was analyzed by flow cytometry using anti-CD4 (e-bioscience 25-0042-82), anti-Vα2 (e-bioscience 47-5812-82) and anti-Vβ5 (e-bioscience 46-5796-82). Cells were acquired on a Fortessa flow cytometer and analyzed using FlowJo software.

Measurement of T Cell Response by Intracellular Staining (Figure 3B). For the detection of IFNγ-producing CD8 T cells by intracellular staining (ICS), mice were sacrificed 5 days after immunization and isolated lymph node cells were set up in 96-well plates at a concentration of 5 × 10^5 cells per well in RPMI medium (Gibco) containing 50 mM 2-ME (Gibco), 10% fetal calf serum (Thermo Hyclone), and antibiotics and incubated with 50 μg/mL of the OVA 323−339 peptide in the presence of BD GolgiPlug (BD Biosciences) for 2 h at 37 °C. Cells were fixed, permeabilized, and stained using the BD Cytofix/Cytoperm kit (BD Biosciences). Intracellular staining (ICS) was performed according to the manufacturer's instructions, with anti-CD4-FITC, anti-CD3-APC-eFluor780, and anti-IFNγ from eBioscience. Cells were acquired on a Fortessa flow cytometer and analyzed using FlowJo software.

Measurement of T Cell Response by ELISA (Figure 3C and Figure 3D). Mice were sacrificed 5 days after immunization, and the splenocytes were cultured in RPMI medium (Gibco) containing 50 mM 2-ME (Gibco), 10% fetal calf serum (Thermo Hyclone), and antibiotics (5 × 10^5 cells per well), in the presence or not of OVA 323−339 (1 μg/mL) at 37 °C for 48 h. IFN-γ and IL-5 levels were measured in the supernatants by ELISA using antibodies against IFN-γ and IL-5 (BD Biosciences).

Immunization of Mice (Figures 4 and 5). Six-week-old female C57BL/6 mice were purchased from Charles River Laboratories. Mice were kept in the Pasteur Institute animal house and supplied with water and food ad libitum. All procedures involving mice were in accordance with the French ethical committee CETEA (Comité d'Ethique en Expérimentation Animale) (Project No. 2013-126). Mice were intramuscularly (IM) or intradermally (ID) immunized with 11.7 nmol of the various compounds on days 0 and 21. Sera were collected at days 1, 21, and 28. In the experiment depicted on Figure S, mice also received a third injection of the compounds at day 92 and their sera were collected at day 111

Analysis of Antibody Response by ELISA (Figures 4 and 5). Sera from immunized mice were tested for the presence of antibodies by ELISA, using MaxiSorp plates (Nunc) coated with the various compounds. For the detection of anti-Tn antibodies, the biotinylated Tn3-G6K(Biot)G peptide (1 μg/mL) was incubated for 1 h at 37 °C on streptavidin-coated microtiter plates (Pierce). Then, serial dilutions of sera were performed and bound Abs were revealed using anti-mouse-IgG-HRP peroxidase conjugate (Sigma) and *o*-phenyldiamine/H_2O_2 substrates. Plates were read photometrically at 492 nm in an ELISA autoreader (Dynatech, Marnes la Coquette, France). The negative control consisted of naive mouse sera diluted 100-fold. ELISA Ab titers were determined by linear regression analysis, plotting dilution vs absorbance at 492 nm. The titers were calculated to be the $\log_{10}$ highest dilution that gave twice the absorbance of normal mouse sera diluted 1/100. Titers were given as the arithmetic mean ± SD of the $\log_{10}$ titers.

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■ ASSOCIATED CONTENT

Supporting Information

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Synthesis of compounds **1** and **2** and TLR7 agonist **24**; HPLC chromatograms for compounds **3**−**8**, **13**, **14**, **29**, **30**, **31**, **33**−**36**; HRMS results for compounds **3**, **4**, **6**, **9**, **10**, **12**, **13**, **15**−**17**, **19**−**22**, **24**, **26**−**36**; HSQC spectra for compounds **3**, **4**, **6**, **9**, **11**, **13**, **15**, **16**, **27**, **29**, **30**, **31**, **33**, **34**, and MALDI-TOF spectra for **35** and **36** (PDF)

Molecular formula strings (CSV)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

Boc, *tert*-butoxycarbonyl; CLR, C-type lectin receptor; CuAAC, copper azide−alkyne cycloaddition; DIEA, *N*,*N*-diisopropylethylamine; DC, dendritic cell; DMF, *N*,*N*-dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; GalNAc, *N*-acetylgalactosamine; HATU, azabenzotriazole-tetramethyluranium hexafluorophosphate; HFIP, hexafluoroisopropanol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IBCF, isobutyl chloroformate; MGL, macrophage galactose-type C-type lectin; Man, mannose; OVA, ovalbumin; NHS, *N*-hydroxysuccinimide; PRR, pattern-recognition receptor; SLE, lupus erythematosus; TACA, tumor-associated carbohydrate antigen; TFA, trifluoroacetic acid; TLR, Toll-like receptor; TF, Thomsen−Friedenreich

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