ELECTRONIC SUPPLEMENTARY INFORMATION

Chemical Synthesis and Immunological Evaluation of New Generation Multivalent Anticancer Vaccines based on a Tn Antigen Analogue

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I. GENERAL INFORMATION

A. MATERIALS AND METHODS

All chemical reagents were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France) or Acros (Noisy-Le-Grand, France) and were used without further purification. All protected amino acids, Rink amide and Fmoc-Gly-Sasrin resins were obtained from Advanced ChemTech Europe (Brussels, Belgium), Bachem Biochimie SARL (Voisins-Les-Bretonneux, France) and France Biochem S.A. (Meudon, France). Progress of the reactions involving carbohydrate reagents was monitored by thin layer chromatography (TLC) using silica gel 60 F254 pre-coated plates (Merck). Spots were visualized by staining with 10% sulfuric acid in ethanol. Silica gel 60 (230-400 mesh, Merck) was used for column chromatography.

Analytical RP-HPLC was performed on a Waters Alliance 2695 separation module, equipped with a Waters 2489 UV/visible detector. Analyses were carried out at 1.23 mL/min (Interchim UPTISPHERE X-SERIE, C_{18}, 5 µm, 125x3.0 mm) with UV monitoring at 214 nm and 250 nm, using a linear A–B gradient [A: 0.1% trifluoroacetic acid (TFA) in water; B: 0.1% TFA in 90% aq. acetonitrile]. Preparative RP-HPLC was performed on a Gilson GX 281 liquid handler equipped with a fraction collector, or on a Waters instrument incorporating a Waters 600 controller and a Waters 2487 Dual Absorbance Detector. Purifications were carried out at a flow rate of 22.0 mL/min (Macherey-Nagel VP, C_{18}, 7 µm, 300 Å, 250x21 mm) with UV monitoring at 214 nm and 250 nm, using a linear A–B gradient. Analytical RP-UPLC (coupled with ESI-MS) was performed on a Waters Acquity UPLC system. Analyses were carried out at 0.6 mL/min (Phenomenex WIDEPORE XB-C18 column, 3.6 µm, 300 Å, 50 × 2.1 mm) with UV monitoring at 214 nm, using a linear C–D gradient (C: 0.1% formic acid in water; D: 0.1% formic acid in acetonitrile).

ESI-MS spectra were recorded on a Waters Acquity UPLC-MS equipped with an SQ Detector 2. HRMS spectra were recorded by ICMG (Institut de Chimie Moléculaire de Grenoble) mass spectrometry (MS) platform on a Waters Xevo G2-S QTof. MALDI-TOF spectra were recorded by ICMG MS platform on a AutoFlex I Bruker after sample pre-treatment in an OligoR3 microcolumn (Applied Biosystems, USA) using a 2,5-dihydroxybenzoic acid matrix.

II. GENERAL SYNTHETIC PROCEDURES

A. Determination of Resin Loading

The Fmoc-resin was weighed, added to the reactor (up to 4 g of resin), and swollen for 10 min twice, first in DCM, then in DMF (10 mL solvent per gram of resin) by stirring with an orbital laboratory agitator (IKA Vibrax VXR basic, USA). After DMF removal, the resin was treated with a 20% piperidine solution in DMF (3 × 10 min) to remove the Fmoc group. The collected deprotection cocktail was transferred to a volumetric flask and made up to the mark. Using a fraction of this solution, the effective loading of the resin was indirectly calculated by measuring the absorption at 299 nm of the corresponding deprotection by-product (dibenzofulvene-piperidine adduct), which relates to the moles of free amino groups per gram of resin according to the following formula:

$$n = \frac{A \cdot V \cdot d \cdot 1000}{l \cdot m \cdot \varepsilon_{299}}$$

with: **n** = free amino groups (mmol/g); **A** = absorbance value at 299 nm; **V** = volumetric flask volume (L); **d** = dilution factor; 1000 = factor conversion of mol to mmol; **l** = optical path length of the cell (cm); **m** = sample weight of resin (g); $\boldsymbol{\varepsilon_{299}}$ = molar attenuation coefficient of dibenzofulvene at 299 nm (7800 L/mol·cm).[1]

B. Solid-Phase Peptide Synthesis (SPPS)

Synthesis of the protected linear peptides was performed manually or in an automated fashion by solid-phase peptide synthesis (SPPS) using the standard 9-fluorenylmethoxycarbonyl (Fmoc)/ *tert*-butyloxycarbonyl (*t*Bu) protection strategy. In manual SPPS, the device consisted of a polypropylene syringe-shaped reactor (150 mL volume) (#5147808 Grace SA, USA), equipped with a filter and a valve in the lower end.

In the manual SPPS protocol, the Fmoc-deprotected resin (20 % piperidine in DMF, 3 × 10 min, see above) was washed several times with DMF (5 × 10 min) before proceeding with the coupling phase. Manual couplings were performed using 1.5–2.0 equiv of the $^{\alpha}$*N*-Fmoc-protected amino acids (Fmoc-Ala-OH, Fmoc-Gly-OH, Fmoc-Lys(Boc)-OH, Fmoc-Pro-OH) activated *in situ* with 1.5–2.0 equiv of PyBOP® and 3.0–4.0 equiv of DIPEA in DMF (10 mL per gram of resin) for 30 min at room temperature (rt). The resin was washed again (5 × 10 min.), and completeness of the amino acid coupling was assessed using the TNBS test.[2] A small portion of resin beads was treated with 2,4,6-trinitrobenzenesulphonic (TNBS) acid (1% w/v solution in DMF), which reacts with free primary amino groups to form an orange-red trinitrophenylated derivative. Following completion of the coupling cycle, the procedure for peptide elongation was repeated from the Fmoc-deprotection step.

Automated peptide synthesis was performed on a 348 Ω Synthesizer (Louisville, KY), using similar conditions to those reported above for the manual SPPS.

C. Peptide Cleavage from Solid Support

Peptide sequences linked to SASRIN™ resin were treated with a TFA/CH_2Cl_2 (1:99, v/v) cleavage cocktail (10 × 10 min). The collected solutions were neutralized with DIPEA and concentrated under reduced pressure. The residue was precipitated with ice-cold diethyl ether, filtered and dried. The resulting white solid was then triturated to give the crude protected peptide as a powder, which was subjected to mass spectrometry analysis.

Peptide sequences linked to Rink amide® resin were treated with a TFA/TIS/H_2O (96:2:2, v/v/v) cleavage cocktail (2 × 3 h). The collected solutions were concentrated under reduced pressure, precipitated with ice-cold diethyl ether, filtered and dried. The resulting white solid was then triturated to give the crude deprotected peptide as a powder, which was subjected to mass spectrometry analysis.

D. Peptide Cyclization

Linear protected peptides were dissolved in CH_2Cl_2/DMF (1:1, ~0.5 mM, based on the calcd. loading) and treated with DIPEA (1.5 equiv) and PyBOP® (1.2 equiv). After stirring the mixture for 30 min, the solvent was removed under vacuum and the crude residue was dissolved in the minimum amount of CH_2Cl_2. To this solution, ice-cold diethyl ether was added, and the crude cyclic peptide was obtained as a white powder after filtration and drying. This crude was then purified through preparative RP-HPLC and lyophilized to give the pure cyclic peptide.

E. Oxime Ligation (OL)

A solution of aldehyde-bearing substrate and aminooxy-functionalized compound (1.2–1.5 equiv per aldehyde) in H_2O containing 0.1% TFA (plus CH_3CN, where necessary) was heated at 37 °C for 30-60 min. When the reaction was completed as assessed by analytical RP-HPLC or UPLC-MS, the crude mixture was purified through preparative HPLC. The fractions containing the desired product were collected and lyophilized to give the oxime-linked product as a white foamy solid.

F. Copper(I)-Catalyzed Alkyne-Azide Cycloaddition (CuAAC)

Three solutions were prepared. Solution A contained the azido-bearing substrate and the alkyne-functionalized compound in DMF. Solution B contained $CuSO_4 \cdot 5H_2O$ (MW 249.69, cat.) and THPTA (MW 434.50, 1–8 equiv per azido group) in PBS buffer (pH 7.4, 10–100 mM). Solution C contained sodium ascorbate (MW 198.11, 5–10 equiv per azido group) in PBS buffer. All solutions were degassed *via* three vacuum/N_2 cycles, then Solution C was added to Solution B, causing the reduction of Cu(II) to Cu(I) and turning the solution color from blue to colorless.

The resulting mixture was added to Solution A, and the reaction was then stirred for 2 h at room temperature. After completion of the reaction as assessed by HPLC or UPLC, a small amount of Chelex® resin (50-100 mesh, Sigma Aldrich) was added to the mixture to chelate copper ions. After stirring for 30 min at room temperature, the reaction mixture was filtered and purified through preparative HPLC to give the triazole-containing product as a white foamy solid after lyophilization.

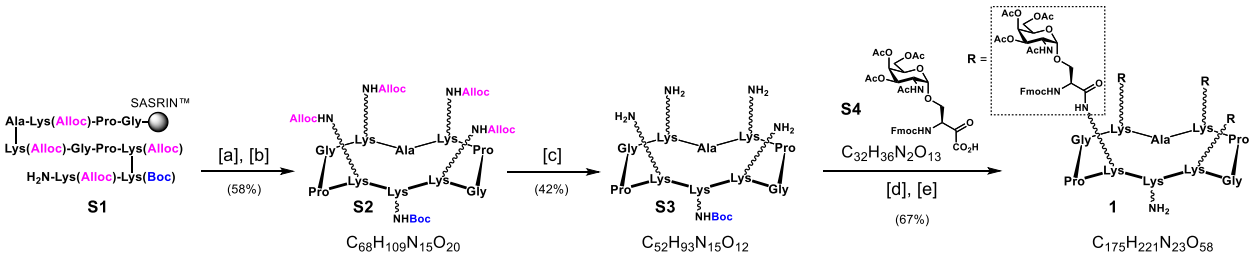
G. Disulfide Bridge Formation

To a solution of Cys(NPys)-containing substrate in a previously degassed NaOAc buffer (pH 4.5, 40 mM) (plus DMF, when required), a solution of cysteine-functionalized peptide (1.1 equiv) in DMF was added. Reaction progress can be monitored visually by the development of an intense yellow coloration of the reaction mixture, indicating cleavage of the NPys moiety. After stirring for 20-30 min at room temperature, reaction completion was assessed by analytical HPLC or UPLC-MS, and the crude was directly purified and lyophilized to give a white foamy solid.

III. SYNTHESIS AND CHARACTERIZATION OF VACCINE CONSTRUCTS

A. SYNTHESIS OF TN-SER CONSTRUCTS

Tn-Ser Protected Tetravalent Glycodendrimer 1.



Scheme S1 Synthesis of Tn-Ser protected tetravalent glycodendrimer **1**. Reagents and conditions: [a] 1% TFA in CH_2Cl_2; [b] PyBOP (1.2 equiv), DIPEA (2.0 equiv), DMF/CH_2Cl_2 (1:1), 0.5 mM, r.t., 30 min, 58% overall yield, [c] $Pd(PPh_3)_4$ (cat.), $PhSiH_3$ (100 equiv), CH_2Cl_2, r.t., 30 min, then MeOH, 42% yield; [d] **S4** (4.8 equiv), DIPEA (6.6 equiv), PyBOP (5.0 equiv), DMF, r.t., 30 min; [e] TFA/CH_2Cl_2 (1:1), r.t., 30 min, 67% yield (over two steps).

Cyclopeptide S2. Following the general synthetic procedure B for SPPS, peptide sequence **S1** was synthesized manually in a polypropylene reactor on a Fmoc-Gly-SASRIN™ resin (2.342 mg, 0.488 mmol/g loading). Cleavage from the solid support was performed according to general synthetic procedure C. Linear peptide **S1** was dissolved in a CH_2Cl_2/DMF (1:1, 3.0 L) mixture in the presence of DIPEA (398 μL, 2.28 mmol, 2.0 equiv) and PyBOP® (715 mg, 1.37 mmol, 1.2 equiv) and compound **S2** (965.6 mg, 58% overall yield) was obtained following general synthetic procedure D for peptide cyclization with RP-HPLC purification using a linear gradient of 5–100% solvent B over 30 min.

HPLC: t_R = 7.75 min (λ_{max} = 214 nm). **HRMS** (ESI^+-TOF) *m/z*: Calcd. for $[M+Na]^+$ 1478.7871 (monoisotopic), found 1478.7893.

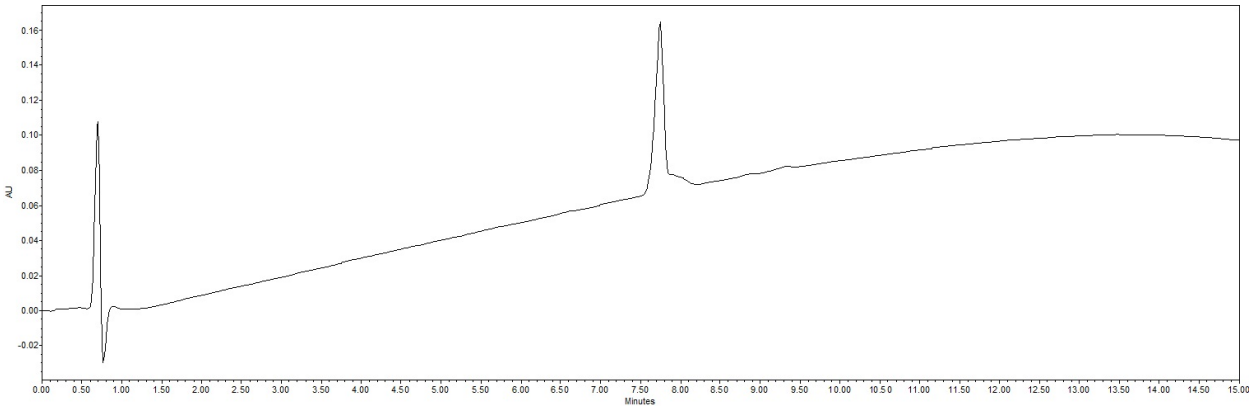


Fig. S1 Analytical RP-HPLC trace of **S2**: t_R = 7.75 min (λ_{max} = 214 nm, 5–100% B over 15 min).

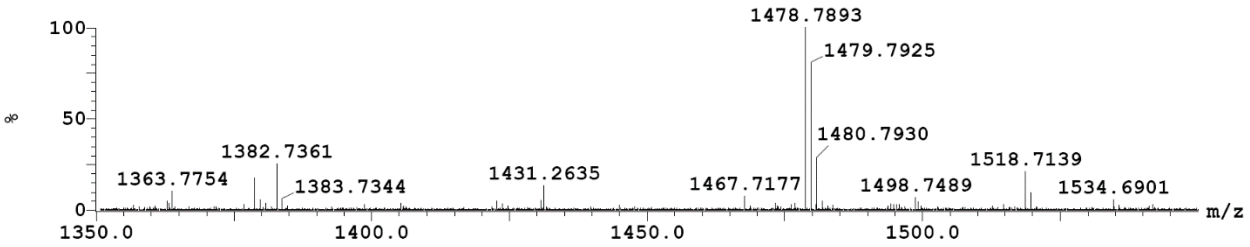


Fig. S2 **HRMS** (ESI$^+$-TOF) spectrum of **S2**: *m/z* calcd. for $[M+Na]^+$ 1478.7871, found 1478.7893.

Cyclopeptide S3. To a stirred solution of **S2** (824.6 mg, 566 μmol, 1.0 equiv) in CH_2Cl_2 (100 mL) $Pd(PPh_3)_4$ (65.4 mg, 56.6 μmol, 0.1 equiv) and $PhSiH_3$ (6.985 mL, 56.611 mmol, 100 equiv) were added. After stirring for 30 minutes at room temperature, MeOH (30 mL) was added, and the reaction was stirred until CO_2 bubbling ceased. The solvent was removed under reduced pressure, the residue was taken up in CH_2Cl_2 (50 mL) and the resulting fine suspension was extracted with H_2O (4 × 50 mL). The combined aqueous fractions were lyophilized, and the obtained solid dissolved in H_2O (10 mL) was purified by RP-HPLC (linear gradient 0–30% solvent B over 15 min). Cyclopeptide **S3** (396.5 mg, 42% yield) was obtained as a white solid after lyophilization.

HPLC: t_R = 9.44 min (λ_{max} = 214 nm). **HRMS** (ESI$^+$-TOF) *m/z*: Calcd. for $[M+H]^+$ 1120.7206 (monoisotopic), found 1120.7244.

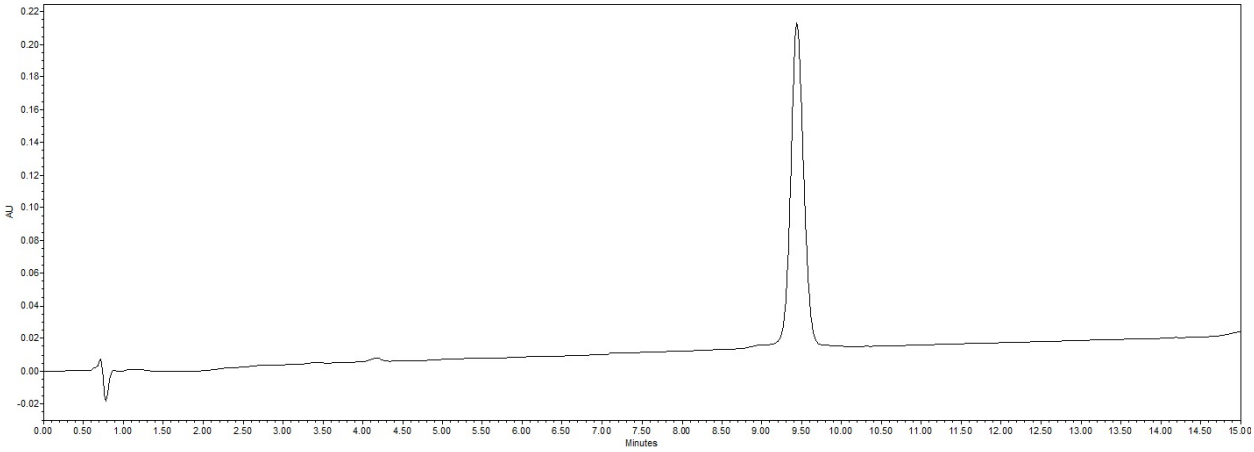


Fig. S3 Analytical RP-HPLC trace of **S3**: t_R = 9.44 min (λ=214 nm, 0–30% B over 15 min).

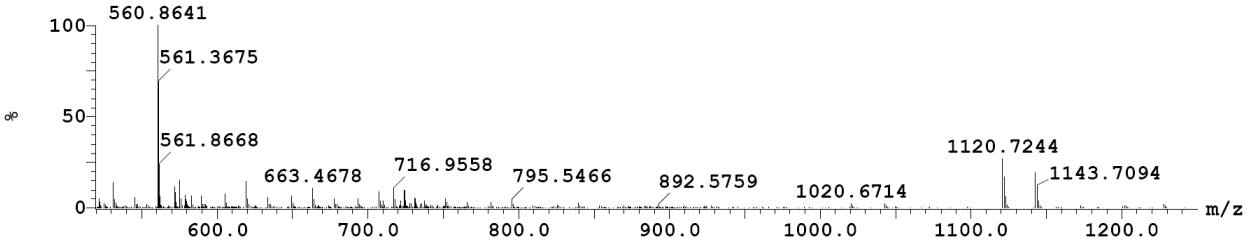


Fig. S4 HRMS (ESI$^+$-TOF) spectrum of **S3**: *m/z* calcd. for $[M+H]^+$ 1120.7206 (monoisotopic), found 1120.7244.

Tn-Ser protected tetravalent glycodendrimer 1. To a solution of **S3** (87.8 mg, 78.4 µmol, 1.0 equiv) in DMF (15.0 mL), DIPEA (90 µL, 516.7 µmol, 6.6 equiv), PyBOP® (205.2 mg, 394.4 µmol, 5.0 equiv) and **S4**[3] (247.1 mg, 376.3 µmol, 4.8 equiv) were added. After stirring for 20 min at room temperature, the solvent was removed under high vacuum and the resulting oily residue was treated with a TFA/CH_2Cl_2 (1:1, 1.0 mL) mixture for 30 min at room temperature. The reaction mixture was then added dropwise to ice-cold diethyl ether (100 mL), and the obtained solid was filtrated and dried to give glycodendrimer **1** (187.8 mg, 67% yield over two steps) after preparative RP-HPLC (linear gradient of 5–100% solvent B over 30 min) and lyophilization.

HPLC: t_R = 12.67 min (λ_{max} = 214 nm). **LRMS** (MALDI-TOF) *m/z*: Calcd. for $[M+Na]^+$ 3597.7 (average), found 3598.7.

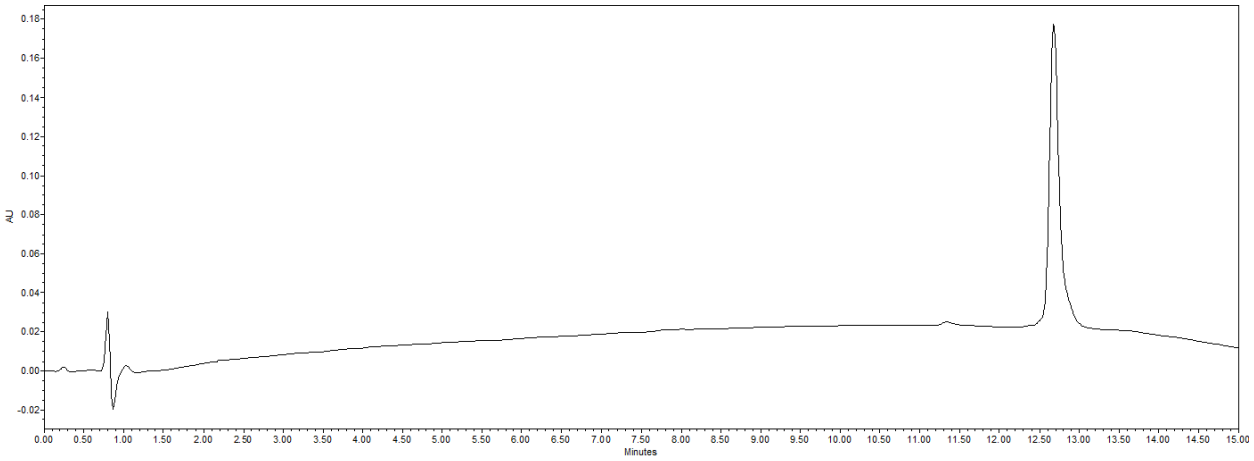


Fig. S5 Analytical RP-HPLC trace of **1**: t_R = 12.67 min (λ_{max} = 214 nm, 5–100% B over 15 min).

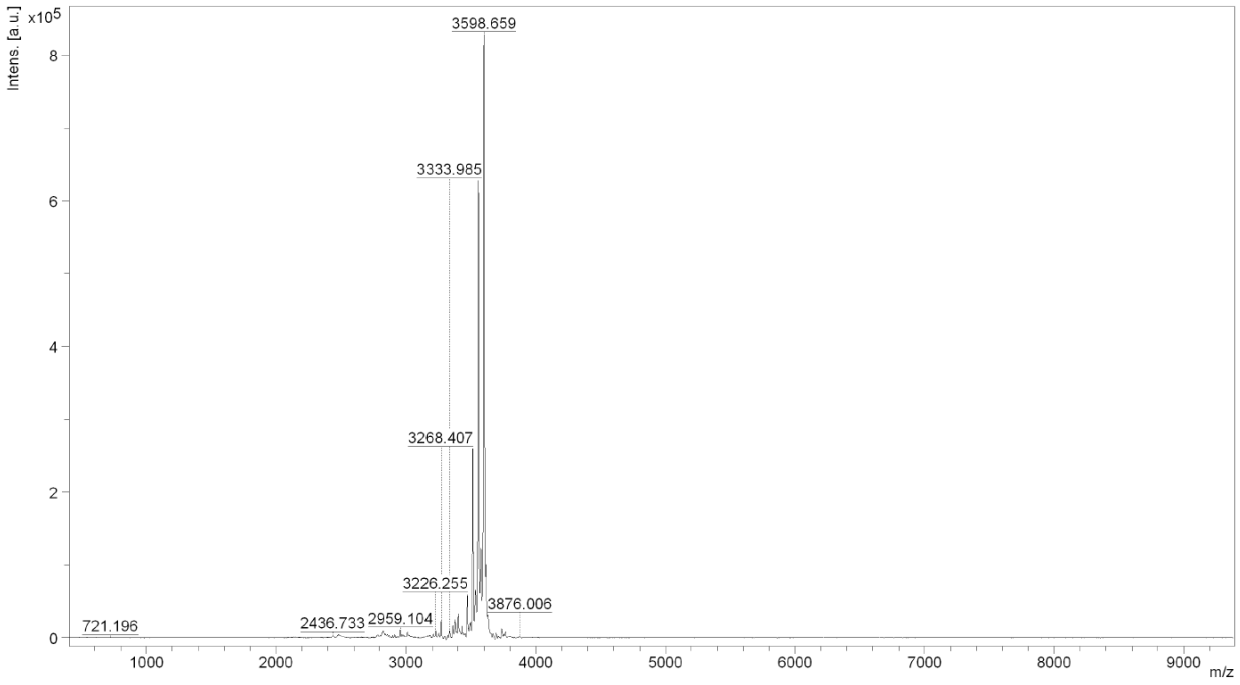
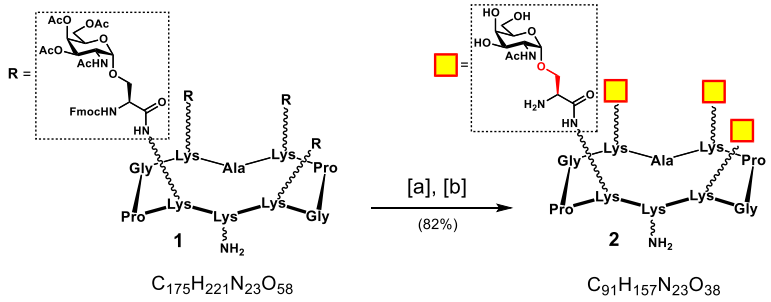


Fig. S6 LRMS (MALDI-TOF) spectrum of glycodendrimer **1**: *m/z* calcd. for $[M+Na]^+$ 3597.7 (average), found 3598.7.

Tn-Ser Tetravalent Glycodendrimer 2.



$C_{175}H_{221}N_{23}O_{58}$ $C_{91}H_{157}N_{23}O_{38}$

Scheme S2 Synthesis of unprotected glycodendrimer **2**. Reagents and conditions: [a] 5% piperidine in CH_3CN, r.t., 15 min; [b] NaOMe/MeOH (pH≈10), r.t., 20 min, 82% yield (over two steps).

Protected glycodendrimer **1** (9.2 mg, 2.6 μmol, 1.0 equiv) was dissolved in piperidine/CH_3CN (5% v/v, 1.0 ml) and the mixture was stirred for 15 min, until complete Fmoc removal as assessed by UPLC-MS. The solvent was evaporated and the white residue was dissolved in dry MeOH (pH≈10, X.x mL) and a NaOMe solution (25 wt.% in methanol) was added. After stirring for 20 min at room temperature, UPLC-MS showed complete deprotection. The reaction mixture was neutralized with Amberlite IR-120 H^+, the resin was filtered off, washed with MeOH and the filtrate was purified by RP-HPLC (linear gradient of 0–20% solvent B over 30 min) to give unprotected glycodendrimer **2** (4.6 mg, 82% overall yield) after lyophilization.

HPLC: t_R = 3.66 min (λ_{max} = 214 nm). **LRMS** (ESI$^+$-MS) *m/z*: Calcd. for $[M+H]^+$ 2181.1 (monoisotopic), found 2180.6.

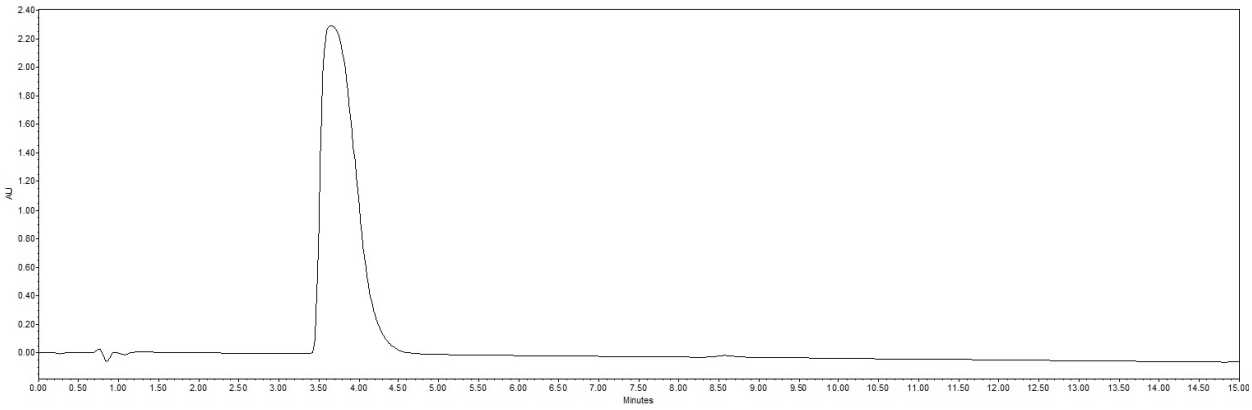


Fig. S7 Analytical RP-HPLC trace of **2**: t_R = 3.66 min (λ_{max} = 214 nm, 0–20% B over 15 min).

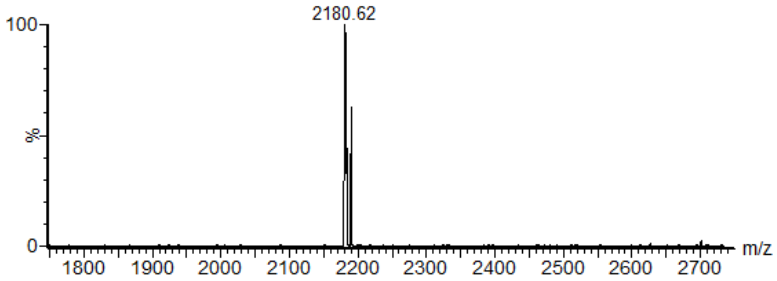
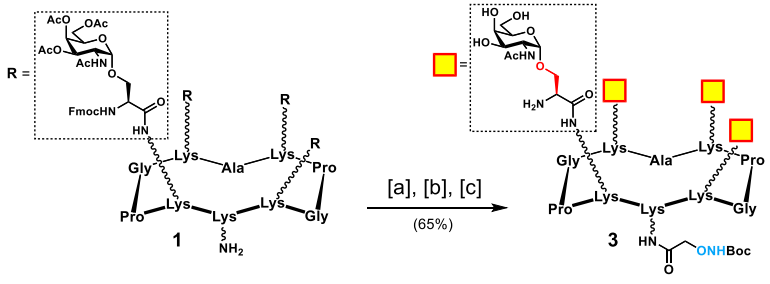


Fig. S8. LRMS (ESI$^+$-MS) spectrum of glycodendrimer **2**: *m/z* calcd. for $[M+H]^+$ 2181.1 (monoisotopic), found 2180.6.

Lys(Boc-Aoa) Tn-Ser Tetravalent Glycodendrimer 3.



Scheme S3 Synthesis of tetravalent glycodendrimer 3. Reagents and conditions: [a] Boc-Aoa-NHS (1.2 equiv), DIPEA (2.0 equiv), CH_3CN/DMF (1:1), 20 min; [b] 5% piperidine in CH_3CN, r.t., 15 min; [c] NaOMe/MeOH (pH≈10), r.t., 20 min, 65% yield (over three steps).

To a solution of glycodendrimer **1** (58.6 mg, 16.4 μmol, 1.0 equiv) in CH_3CN/DMF (1:1, 3.5 mL), DIPEA (5.7 μL, 32.7 μmol, 2.0 equiv) and Boc-aminooxyacetic acid N-hydroxysuccinimide ester (Boc-Aoa-NHS)[4] (5.7 mg, 19.8 μmol, 1.2 equiv) were added. After stirring for 20 minutes at room temperature the solvent was evaporated and the resulting residue was used in the next reaction without further purification.

The crude was treated with piperidine/CH_3CN (5% v/v, 2.0 mL) for 15 minutes at room temperature and the mixture was concentrated to give a white residue. This crude was dissolved in dry MeOH (pH≈10, 5.0 mL) and then treated with NaOMe (25 wt.% solution in methanol) for 20 min at room temperature until UPLC-MS showed complete deprotection. The reaction mixture was neutralized with Amberlite IR-120 H^+, the resin was filtered off, washed with MeOH, and the filtrate was purified by RP-HPLC (linear gradient of 0–40% solvent B over 30 min) to give glycodendrimer **3** (25.1 mg, 65% overall yield) after lyophilization.

HPLC: t_R = 8.37 min. (λ_{max} = 214 nm). **HRMS** (ESI$^+$-TOF) *m/z*: Calcd. for $[M+H]^+$ 2354.1826 (monoisotopic), found 2354.1836.

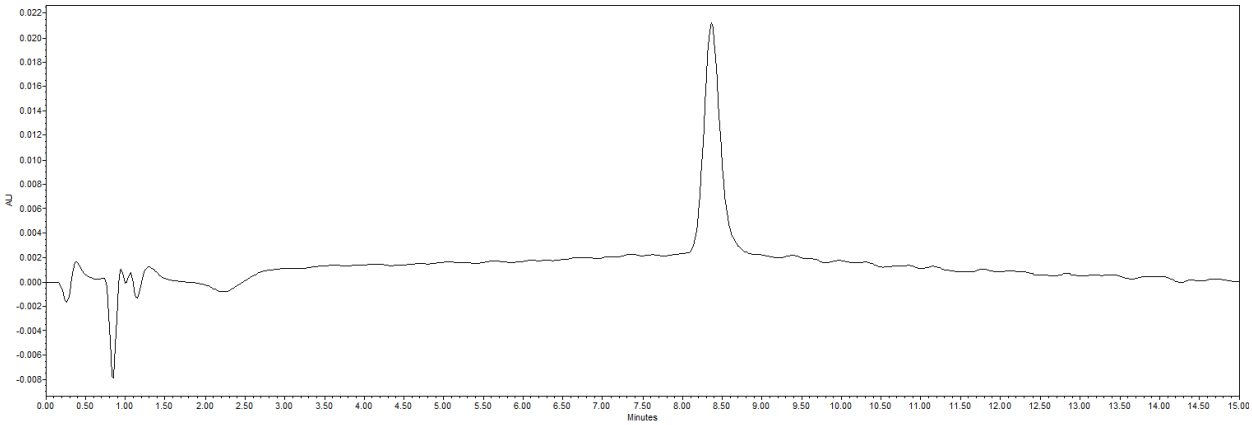


Fig. S9 Analytical RP-HPLC trace of **3**: t_R = 8.37 min (λ_{max} = 214 nm, 0–40% B over 15 min).

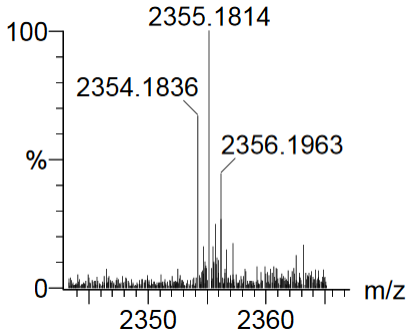
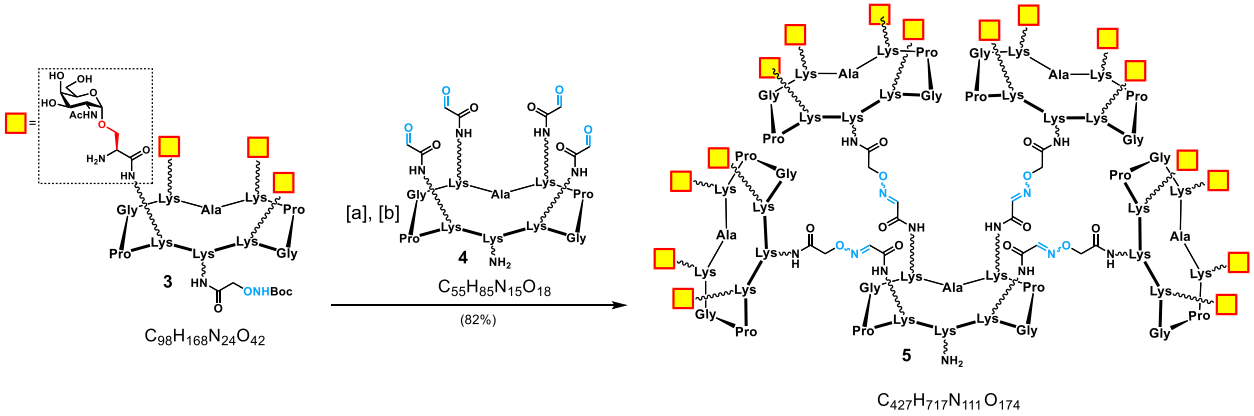


Fig. S10 HRMS (ESI$^+$-TOF) of **3**: *m/z* calcd. for $[M+H]^+$ 2354.1826 (monoisotopic), found 2354.1836.

Tn-oxime Hexadecavalent Glycodendrimer 5.



Scheme S4 Synthesis of hexadecavalent glycodendrimer **5**. Reagents and conditions: [a] TFA/CH_2Cl_2 (1:1), r.t., 30 min; [b] **4**[5] (0.17 equiv), 0.1% TFA in H_2O, 37 ºC, 45 min, 82% yield (over two steps).

Glycodendrimer **3** (29.7 mg, 12.6 μmol, 1.0 equiv) was treated with TFA/CH_2Cl_2 (1:1, 1.0 mL) for 30 min at room temperature. The reaction mixture was added dropwise to ice-cold diethyl ether (20 mL), the obtained solid was filtered, dried and used in the next step without further purification. The crude solid was reacted with **4**[5] (1.7 mg, 2.1 μmol, 0.17 equiv) in H_2O (0.1% TFA, 1.0 mL), following the general procedure E for oxime ligation. Hexadecavalent glycodendrimer **5** (17.5 mg, 82% overall yield) was obtained after RP-HPLC purification (linear gradient of 0–40% solvent B over 30 min) and lyophilization.

HPLC: t_R = 8.67 min. (λ_{max} = 214 nm). **LRMS** (MALDI-TOF) *m/z*: Calcd. for $[M+H]^+$ 10190.9 (average), found 10194.5.

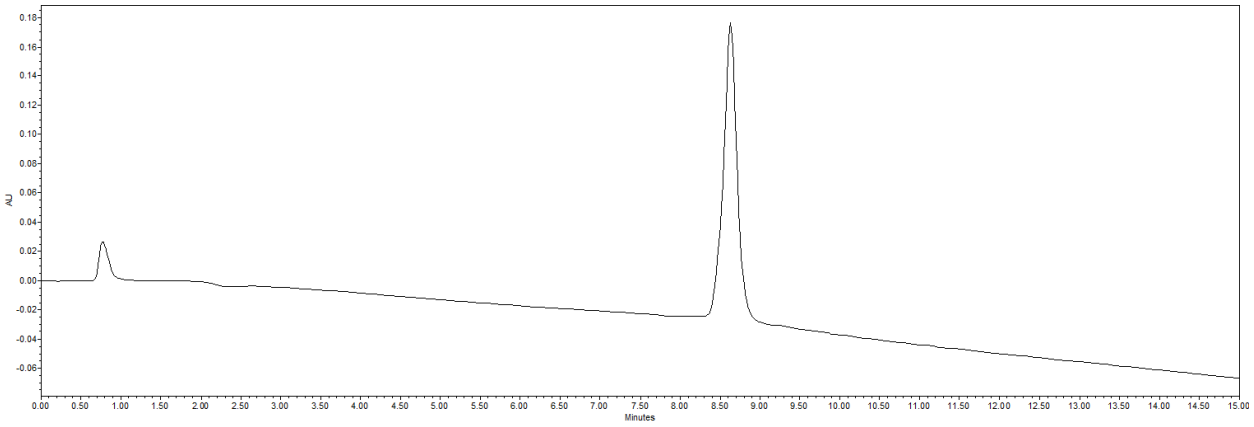


Fig. S11 Analytical RP-HPLC trace of **5**: t_R = 8.67 min (λ_{max} = 214 nm, 0–40% B over 15 min).

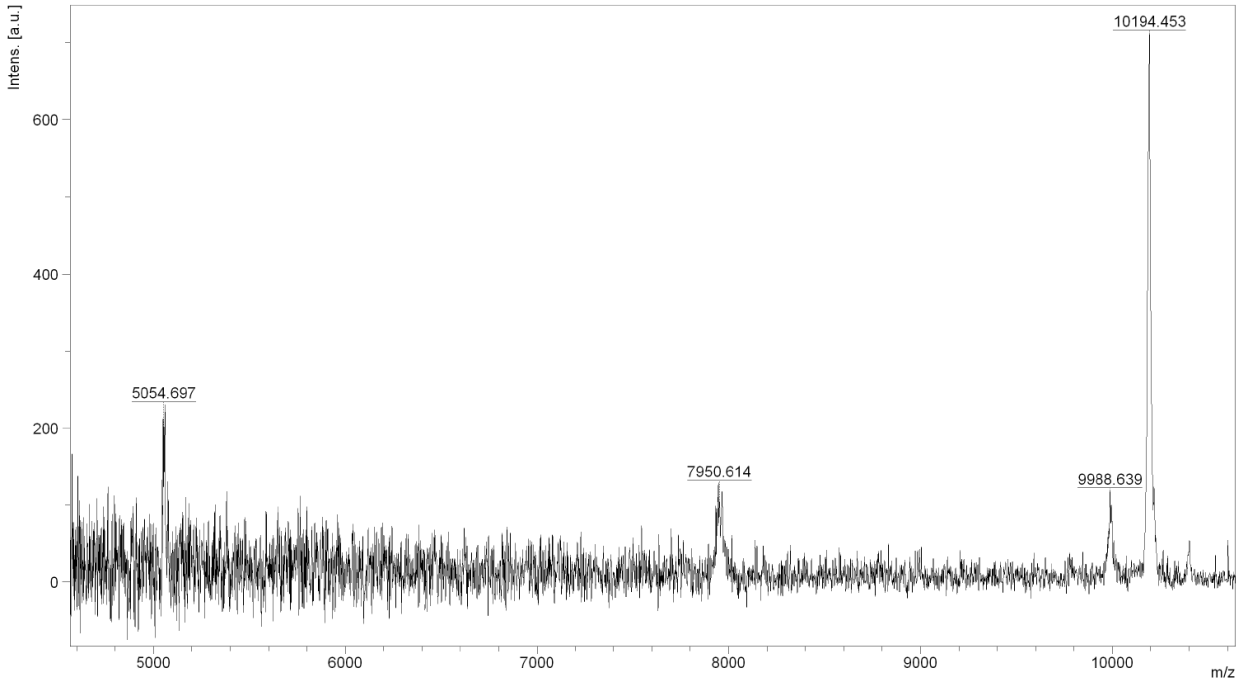
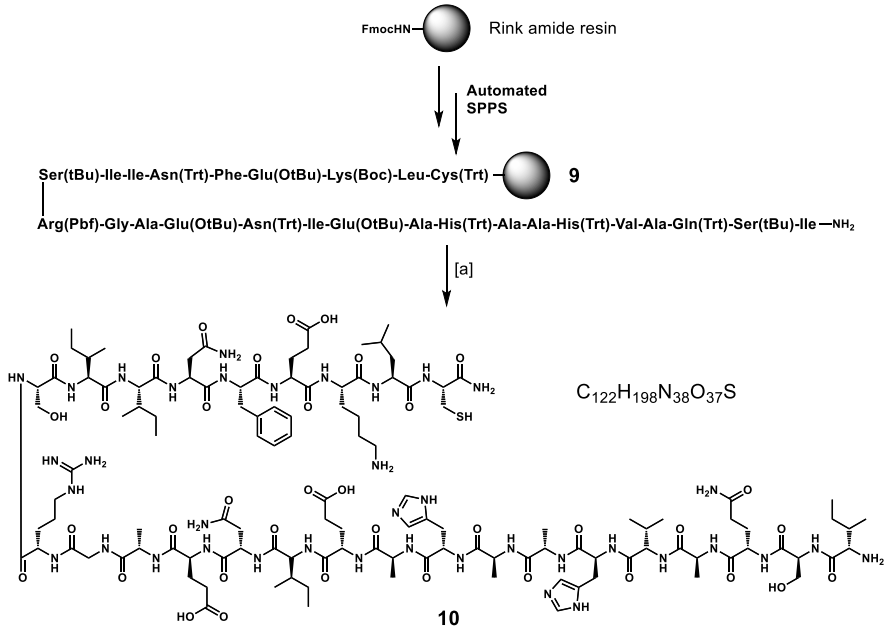


Fig. S12 LRMS (MALDI-TOF) spectrum of **5**: *m/z* calcd. for $[M+H]^+$ 10190.9 (average), found 10194.5.

CD4$^+$-CD8$^+$ OVA Peptide 10.



Scheme S5 Synthesis of OVA peptide **10**. Reagents and conditions: [a] TFA/TIS/H_2O (96:2:2), r.t., 3 h (× 3), 41 % overall yield.

Peptide sequence **9** was synthesized by SPPS according to the general synthetic procedure B on a Rink amide® resin (535 mg, 0.524 mmol/g loading). Cleavage from the solid support was performed following general synthetic procedure C and the crude peptide was purified by RP-HPLC (linear gradient of 0–40% solvent B over 30 min) to give fully deprotected OVA peptide **10** (324.2 mg, 41% yield) after lyophilization.

HPLC: t_R = 7.24 min. (λ_{max} = 214 nm). **HRMS** (ESI$^+$-TOF) *m/z*: Calcd. for $[M+H]^+$ 2821.4607 (most intense peak of isotopic cluster), found 2821.4568.

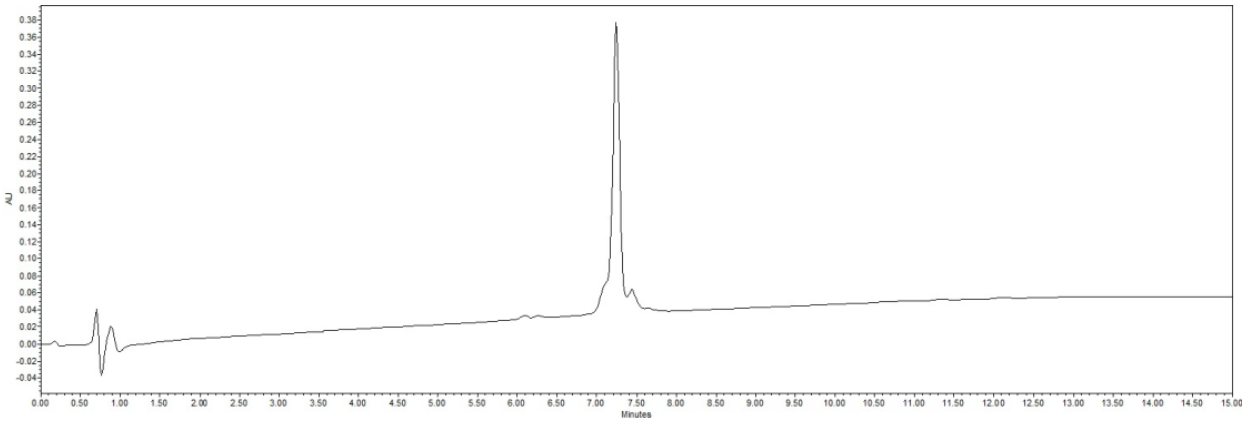


Fig. S13 Analytical RP-HPLC trace of **10**. t_R = 7.24 min (λ_{max} = 214 nm, 0–40% B over 15 min)

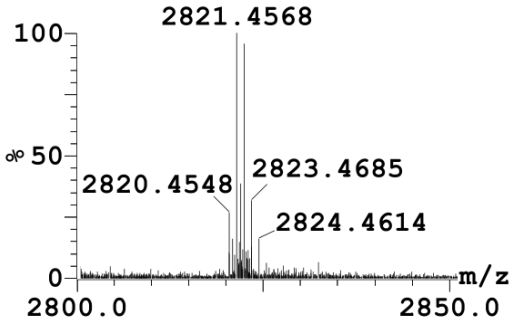
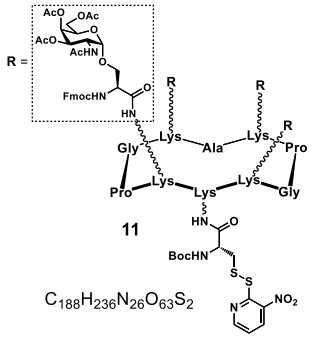


Fig. S14 HRMS (ESI$^+$-TOF) of **10**: *m/z* calcd. for $[M+H]^+$ 2821.4607 (monoisotopic), found 2821.4568.

Boc-Cys(NPys) Tn-Ser Protected Tetravalent Glycodendrimer 11.



To a solution of glycodendrimer **1** (13.2 mg, 3.7 μmol, 1.0 equiv) in DMF (1.0 mL), DIPEA (0.97 μL, 5.6 μmol, 1.5 equiv) and Boc-Cys(NPys)-NHS[6] (2.6 mg, 5.5 μmol, 1.5 equiv) were added and the reaction mixture was stirred for 30 min at room temperature. The crude was directly purified by RP-HPLC (linear gradient of 5–100% solvent B over 30 min) and lyophilized to give functionalized glycodendrimer **11** (11.2 mg, 77% yield) after lyophilization.

HPLC: t_R = 12.62 min. (λ_{max} = 214 nm). **LRMS** (MALDI-TOF) *m/z*: Calcd. for $[M+Na]^+$ 3955.1 (average), found 3954.6.

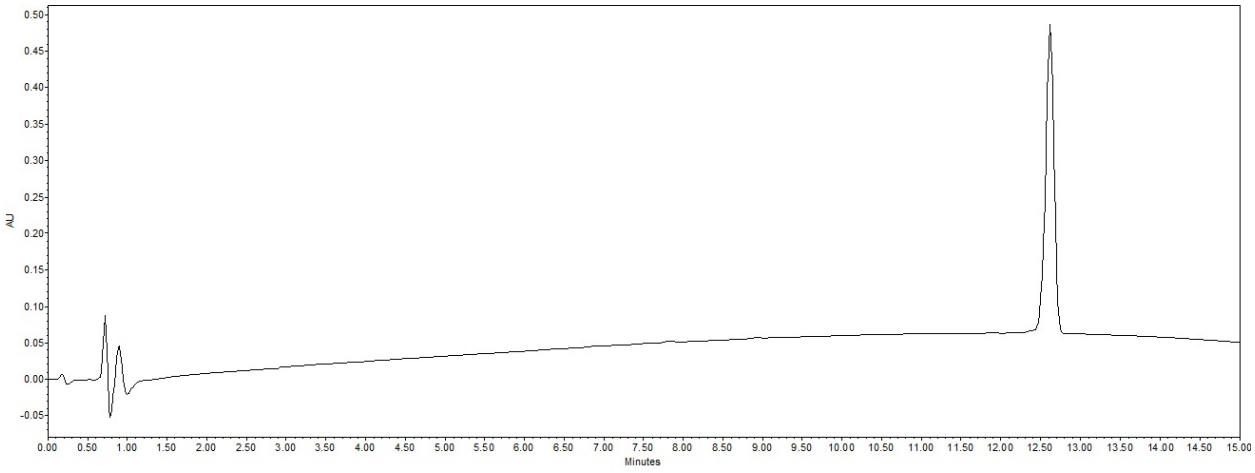


Fig. S155 Analytical RP-HPLC trace of **11**: t_R = 12.62 min (λ_{max} = 214 nm, 5–100% B over 15 min)

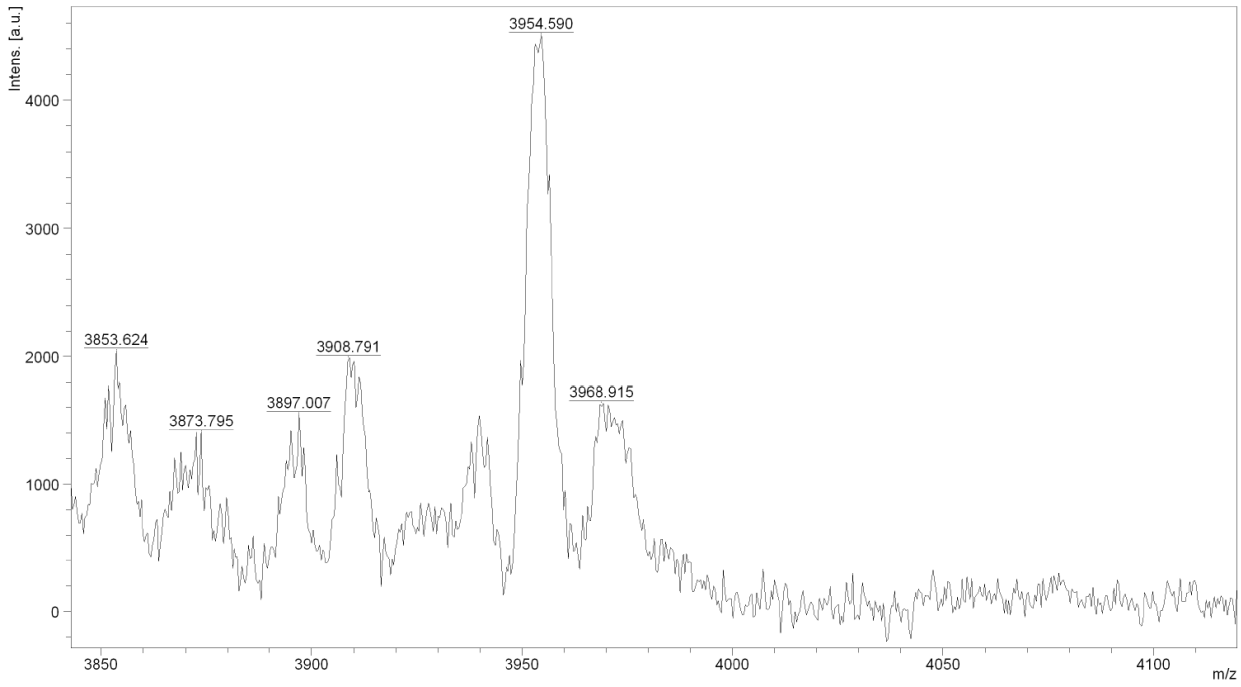
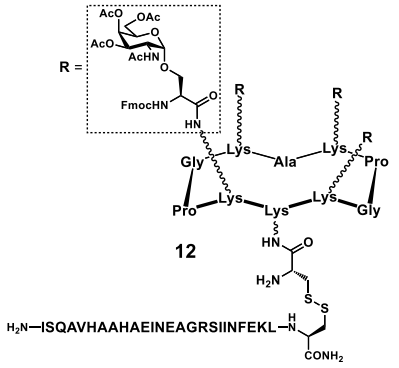


Fig. S166 LRMS (MALDI-TOF) spectrum of **11**: m/z calcd. for $[M+Na]^+$ 3955.1 (average), found 3954.6.

OVA–Tn-Ser Protected Tetravalent Construct 12.



$C_{300}H_{422}N_{62}O_{96}S_2$.

Glycodendrimer **11** (9.5 mg, 2.4 μmol, 1.0 equiv) was treated with TFA/CH_2Cl_2 (1:1, 1.2 mL) for 30 min at room temperature. The reaction mixture was added dropwise to ice-cold diethyl ether (15 mL), and the obtained solid was filtered, dried and used in the next step without further purification. Following general synthetic procedure G for disulfide bridging, the crude was dissolved in DMF/NaOAc buffer (pH 4.5, 40 mM) (2:1, 1.5 mL), and a solution of OVA peptide **10** (7.4 mg, 2.6 μmol, 1.1 equiv) in DMF (500 μL) was added. Protected construct **12** (10.8 mg, 69% overall yield) was obtained after RP-HPLC purification (linear gradient 5–100% solvent B over 30 min) and lyophilization.

HPLC: t_R = 10.59 min (λ_{max} = 214 nm). **LRMS** (MALDI-TOF) m/z: Calcd. for $[M+H]^+$ 6498.1 (average), found 6498.9.

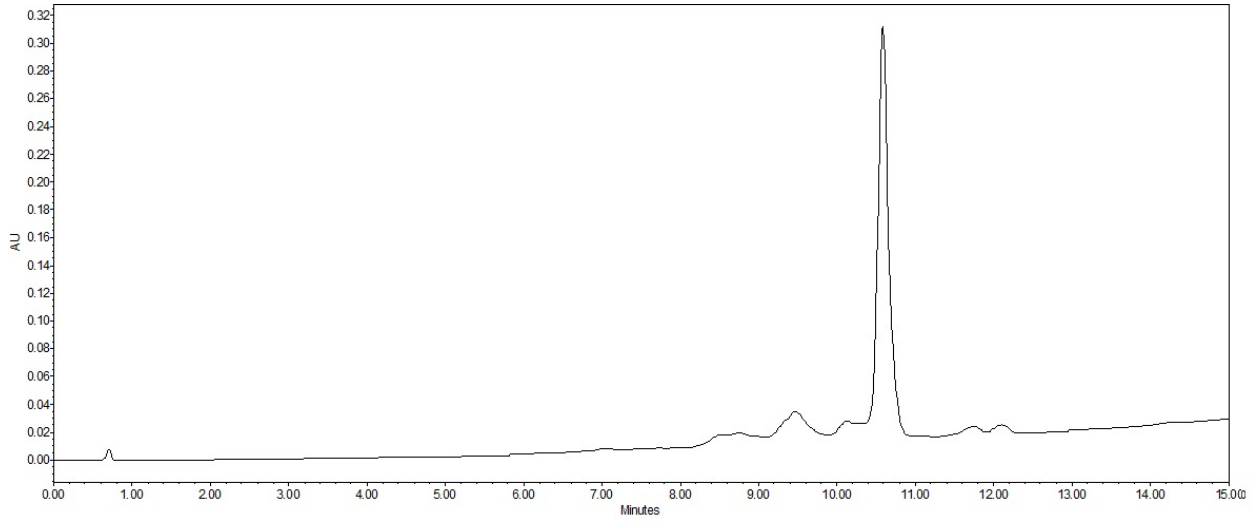


Fig. S177 Analytical RP-HPLC trace of **12**: t_R = 10.59 min (λ_{max} = 214 nm, 5–100% B over 15 min)

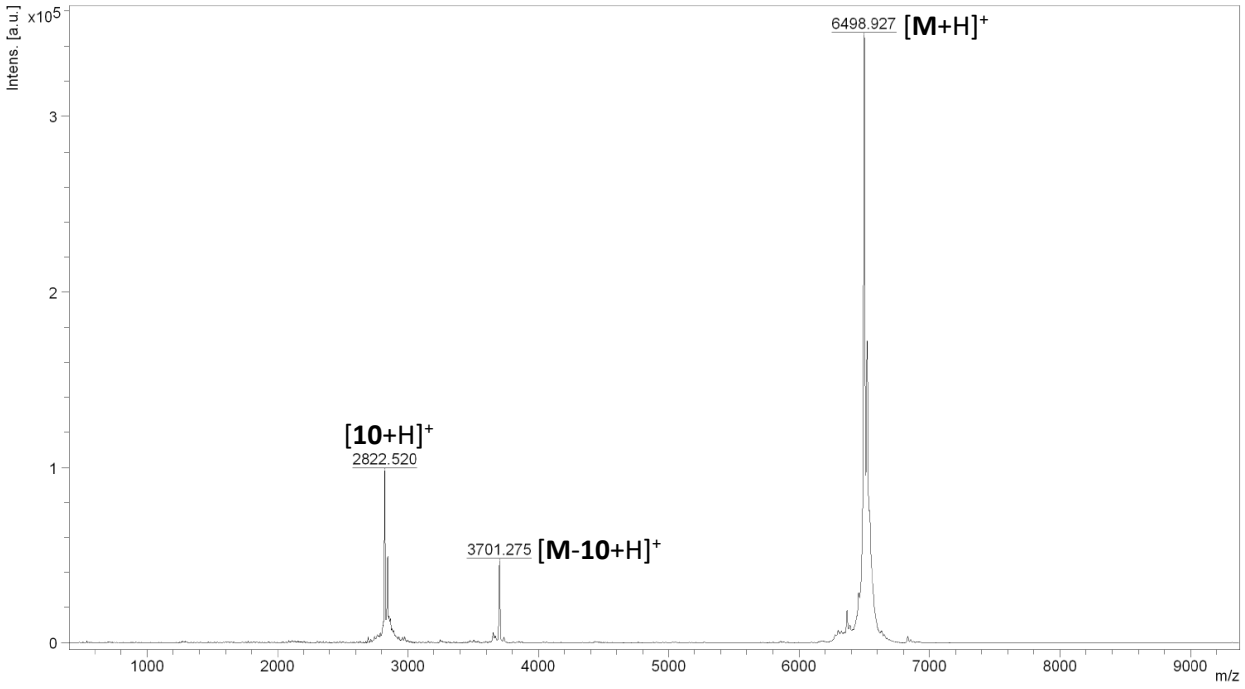
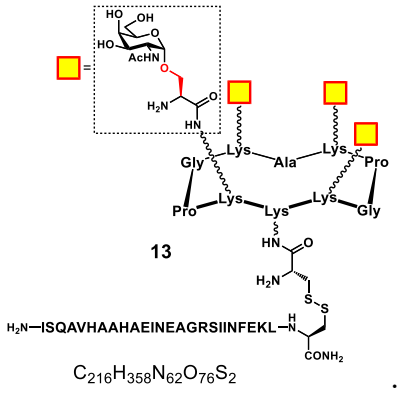


Fig. S18 LRMS (MALDI-TOF) spectrum of **12**: *m/z* calcd. for $[M+H]^+$ 6498.1 (average), found 6498.9. Under MALDI conditions, disulfide bridge is partially cleaved, releasing starting OVA peptide **10** along with the glycodendrimer counterpart.[7]

OVA–Tn-Ser Tetravalent Vaccine Construct 13.



Protected vaccine construct **12** (7.2 mg, 1.1 μmol) was dissolved in dry MeOH (pH≈10, 3.0 mL) and a NaOMe solution (25 wt.% in methanol) was added. The reaction mixture was stirred overnight at room temperature and then neutralized with Amberlite IR-120 H^+. The resin was filtered off, washed with MeOH and the filtrate purified by RP-HPLC (linear gradient of 5–80% solvent B over 30 min) to give vaccine construct **13** (2.4 mg, 43% yield) after lyophilization.

HPLC: t_R = 5.95 min. (λ_{max} = 214 nm). **LRMS** (MALDI-TOF) *m/z*: Calcd. for $[M+H]^+$ 5104.7 (average), found 5104.8.

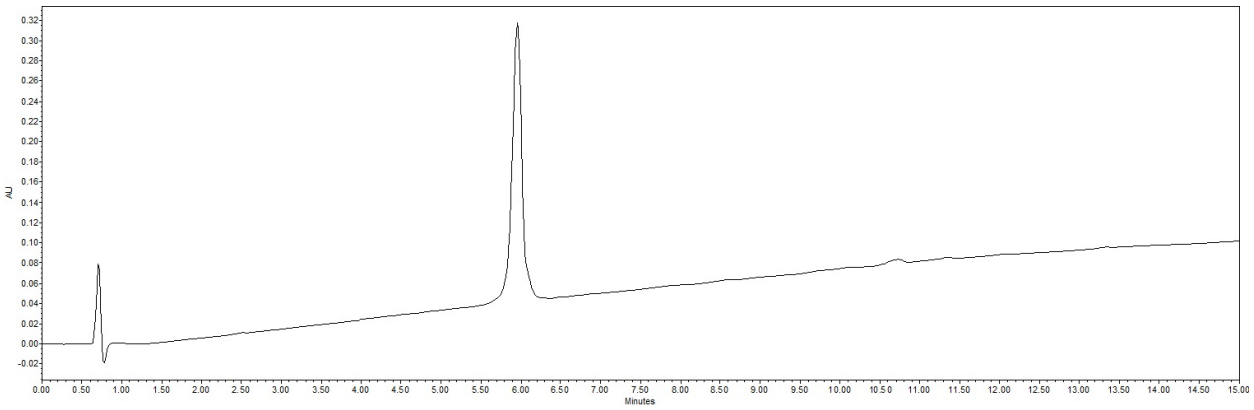


Fig. S19 Analytical RP-HPLC trace of **13**: t_R = 5.95 min (λ_{max} = 214 nm, 5–80% B over 15 min).

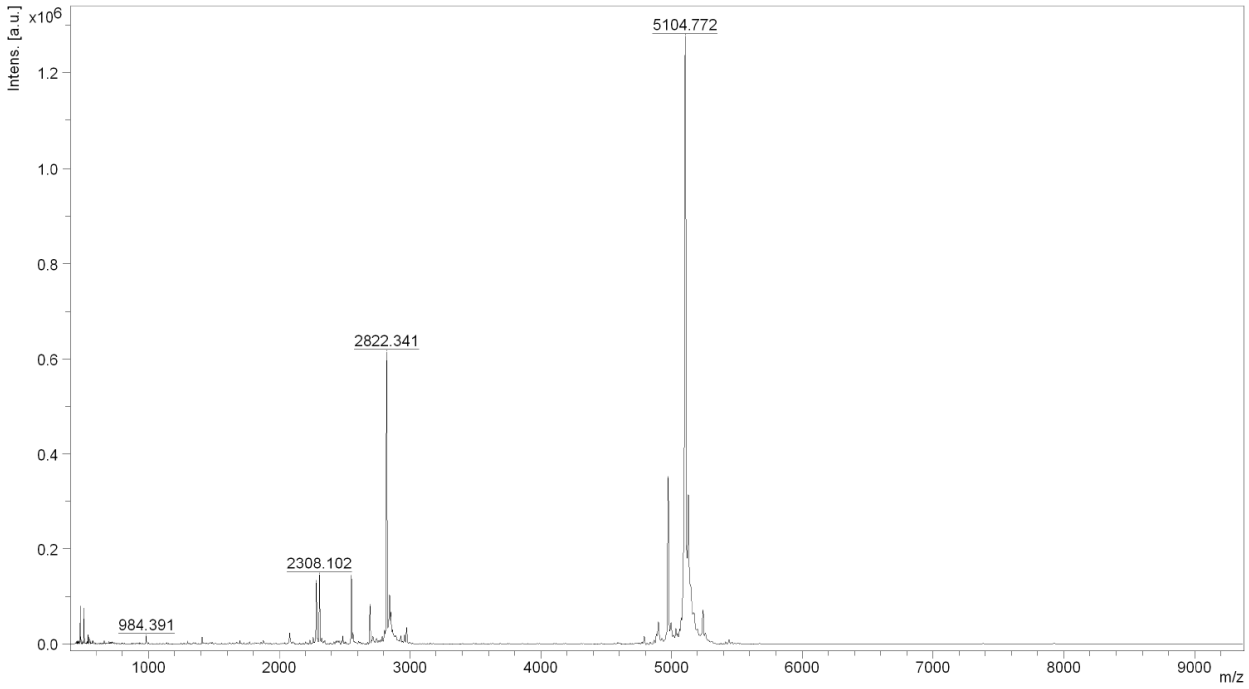
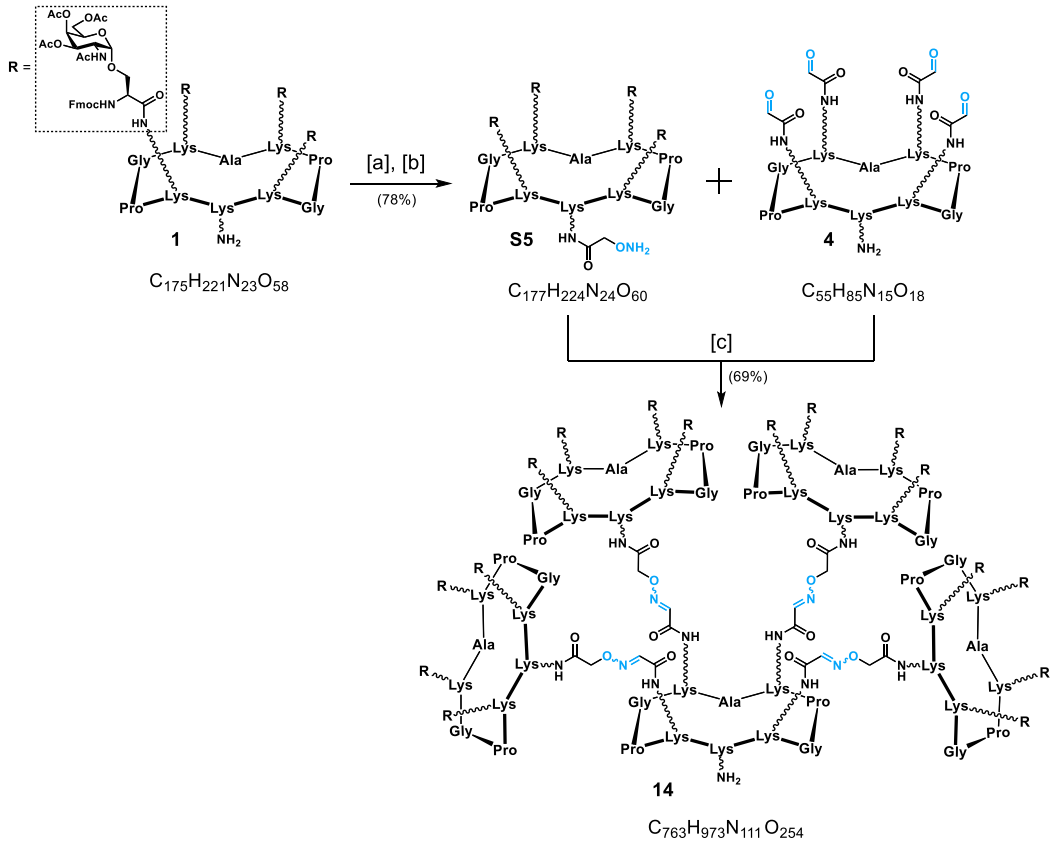


Fig. S20 LRMS (MALDI-TOF) spectrum of **13**: *m/z* calcd. for $[M+H]^+$ 5104.7 (average), found 5104.8.

Tn-Ser Protected Hexadecavalent Oxime-linked Glycodendrimer 14.



Scheme S6 Synthesis of hexadecavalent glycodendrimer **14**. Reagents and conditions: [a] Boc-Aoa-NHS[4] (1.2 equiv), DIPEA (1.5 equiv), DMF, r.t., 30 min; [b] TFA/NH_2OH/CH_2Cl_2 (50:2:48), r.t., 30 min, 78% yield (over two steps); [c] S5 (6.0 equiv), 4 (1.0 equiv), 0.1% TFA in H_2O/CH_3CN (1:9), 37 ºC, 60 min, 69% yield.

Lys(Aoa) Tn-Ser protected tetravalent glycodendrimer S5. To a solution of glycodendrimer **1** (22.6 mg, 6.3 µmol, 1.0 equiv) in DMF (3.0 mL), DIPEA (1.6 µL, 9.2 µmol, 1.5 equiv) and Boc-Aoa-NHS[4] (2.2 mg, 7.6 µmol, 1.2 equiv) were added. After stirring for 30 min at room temperature, the reaction mixture was concentrated and the crude was treated with TFA/NH_2OH/CH_2Cl_2 (50:2:48, 2.0 mL) for 30 min at room temperature. The reaction mixture was added dropwise to ice-cold diethyl ether (30 mL), and the resulting solid was filtrated and purified by RP-HPLC (linear gradient 5–100% solvent B over 15 min) to give glycodendrimer **S5** (17.9 mg, 78% yield) after lyophilization.

HPLC: t_R = 11.01 min (λ_{max} = 214 nm). **LRMS** (ESI^+-MS) *m/z*: Calcd. for $[M+2H]^{2+}$ 1824.9 (average), found 1825.1; calcd. for $[M+3H]^{3+}$ 1216.9, found 1217.4.

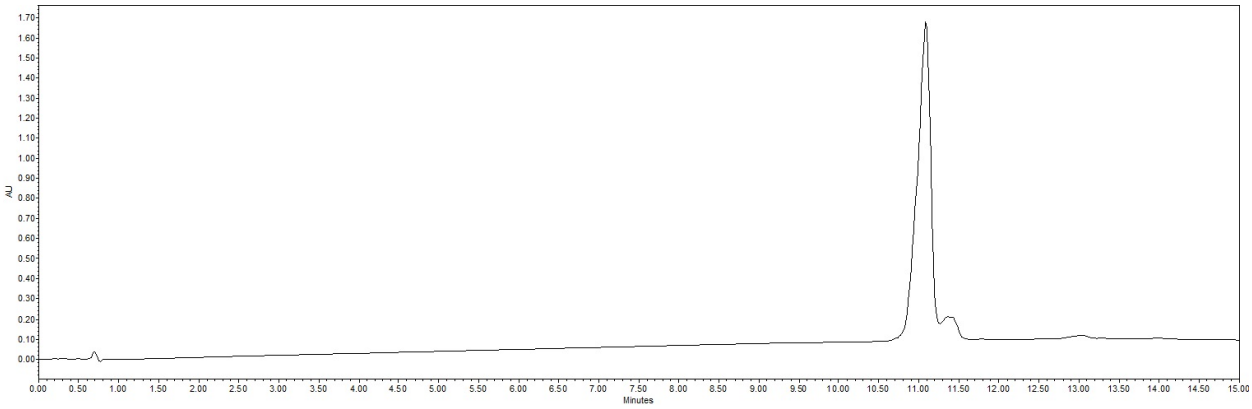


Fig. S181 Analytical RP-HPLC trace of **S5**: t_R = 11.01 min (λ_{max} = 214 nm, 5–100% B over 15 min).

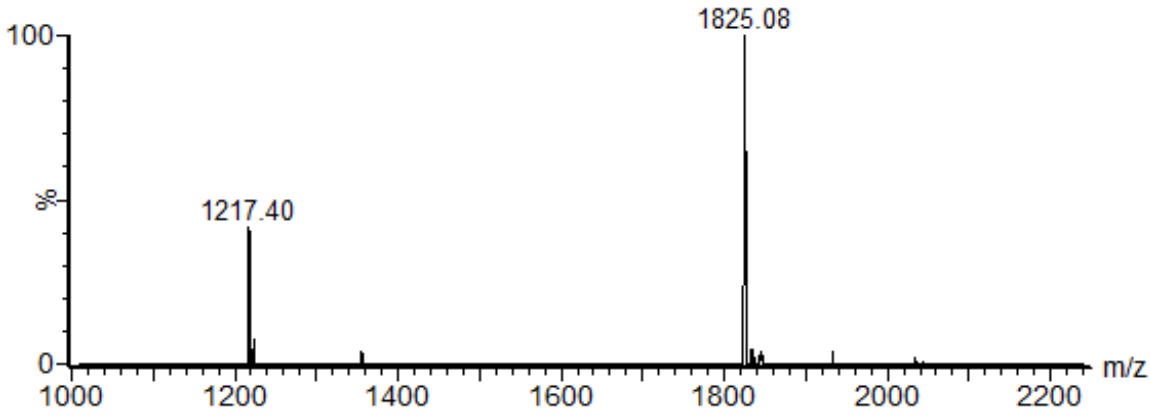


Fig. S22 LRMS (ESI^+-MS) spectrum of **S5**: *m/z* calcd. for $[M+2H]^{2+}$ 1824.9 (average), found 1825.1; calcd. for $[M+3H]^{3+}$ 1216.9, found 1217.4.

Tn-Ser protected hexadecavalent oxime-linked glycodendrimer 14. Following general synthetic procedure E for oxime ligation, oxo-aldehyde-bearing scaffold **4** (0.87 mg, 0.70 µmol, 1.0 equiv) was reacted with aminooxy-functionalized glycodendrimer **S5** (15.42 mg, 4.23 µmol, 6.0 equiv) in CH_3CN/H_2O (9:1, 1.0 mL) containing 0.1% of TFA. Hexadecavalent glycodendrimer **14** (7.61 mg, 69% yield) was obtained after RP-HPLC purification (linear gradient 50–100% over 15 min) and lyophilization.

HPLC: t_R = 12.51 min (λ_{max} = 214 nm). **LRMS** (MALDI-TOF) *m/z*: Calcd. for $[M+Na]^+$ 15786.5 (average), found 15789.1.

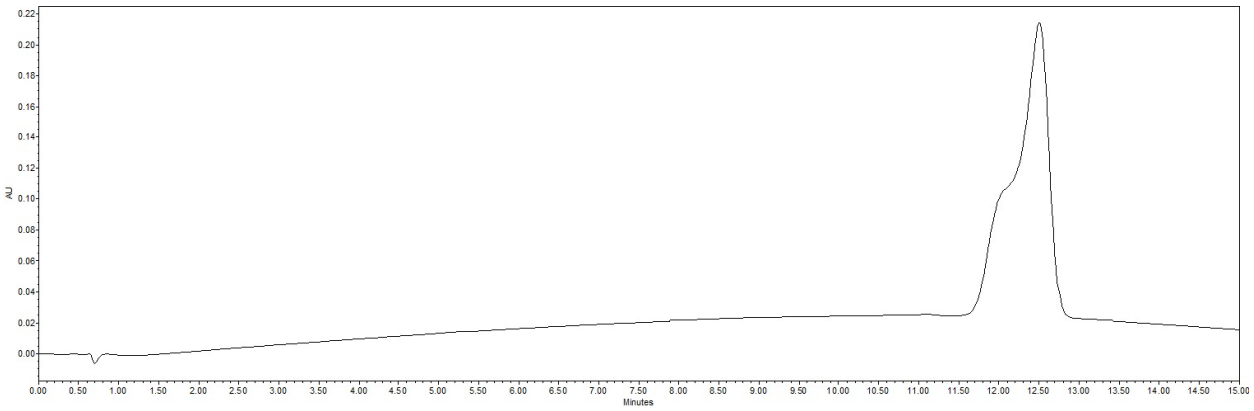


Fig. S193 Analytical RP-HPLC trace of **14**: t_R = 12.51 min (λ_{max} = 214 nm, 50–100% B over 15 min).

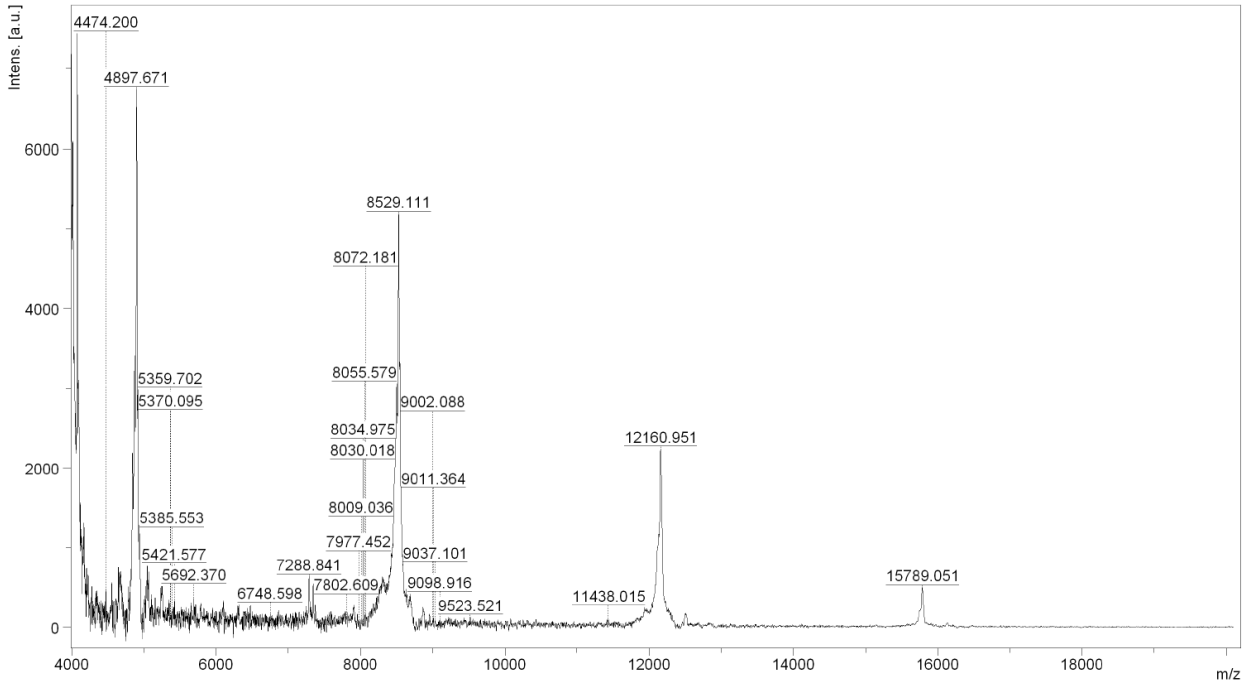
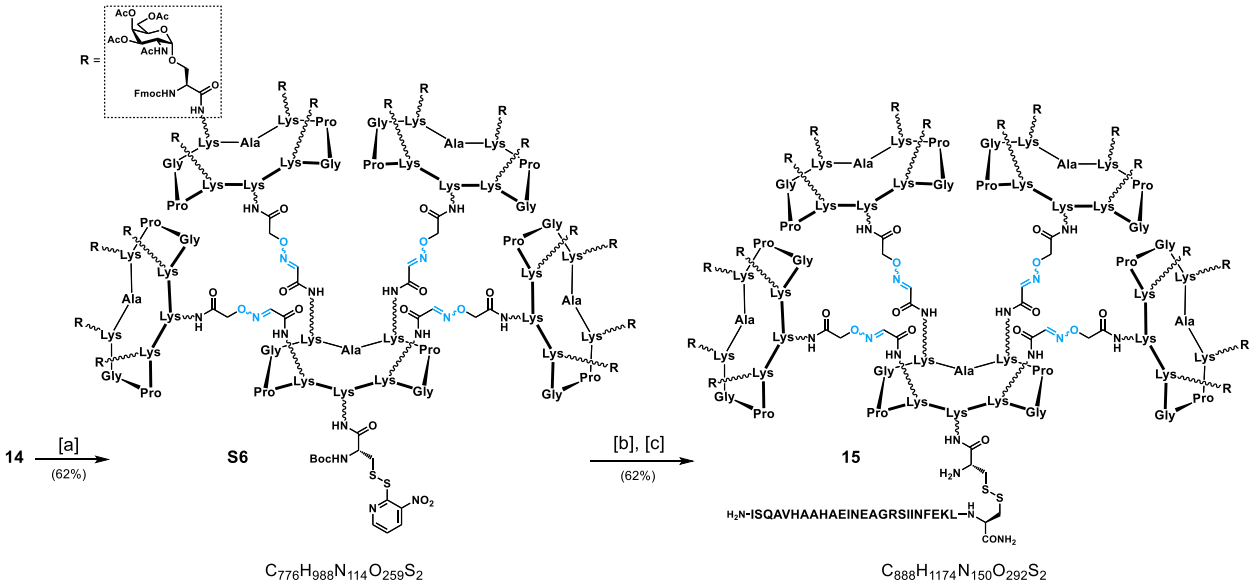


Fig. S204 LRMS (MALDI-TOF) spectrum of **14**: *m/z* calcd. for $[M+Na]^+$ 15786.5 (average), found 15789.1.

OVA–Tn-Ser Protected Hexadecavalent Oxime-linked Construct 15.



Scheme S7 Synthesis of hexadecavalent glycodendrimer **15**. Reagents and conditions: [a] Boc-Cys(NPys)-NHS[7] (1.5 equiv), DIPEA (2.15 equiv), DMF, r.t., 30 min, 62% yield; [b] TFA/CH_2Cl_2 (1:1), r.t., 30 min; [c] Peptide **10** (1.1 equiv), DMF/NaOAc buffer (pH 4.5, 40 mM) (2:1), r.t., 30 min, 62% yield (over two steps)

Boc-Cys(NPys) Tn-Ser protected hexadecavalent glycodendrimer S6. To a solution of glycodendrimer **14** (6.24 mg, 0.40 µmol, 1.0 equiv) in DMF (1.0 mL), DIPEA (0.15 µL, 0.86 µmol, 2.15 equiv) and Boc-Cys(NPys)-NHS (0.28 mg, 0.59 µmol, 1.5 equiv) were added. The reaction mixture was stirred for 30 min at room temperature and directly purified by RP-HPLC (linear gradient 50–100% over 30 min) to give glycodendrimer **S6** (3.40 mg, 62% yield) after lyophilization.

HPLC: t_R = 12.63 min (λ_{max} = 214 nm). **LRMS** (ESI$^+$-MS) *m/z*: Calcd. for $[M+6H]^{6+}$ 2686.1 (monoisotopic), found 2686.1.

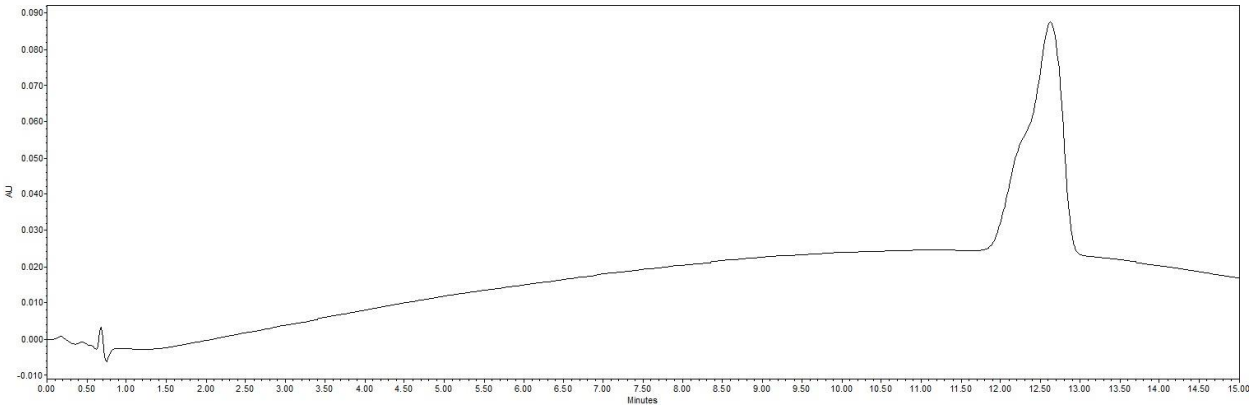


Fig. S215 Analytical RP-HPLC trace of **S6**: t_R = 12.51 min (λ_{max} = 214 nm, 50–100% B over 15 min).

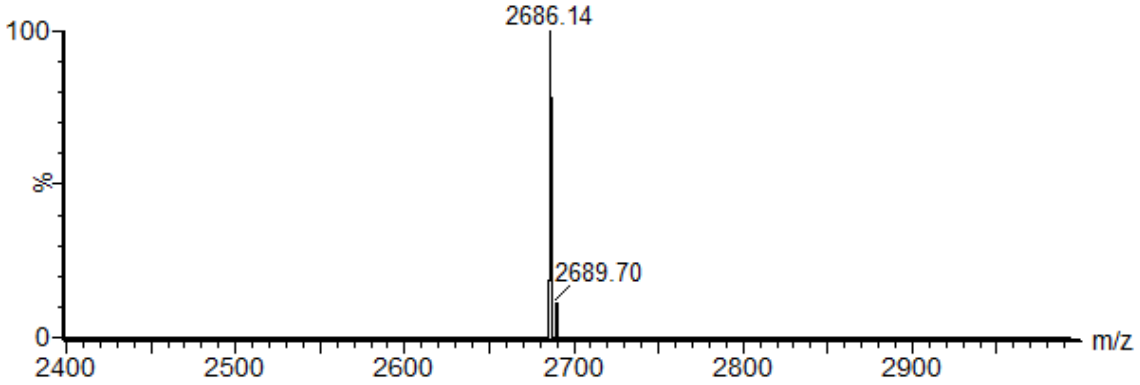


Fig. S26 LRMS (ESI$^+$-MS) spectrum of **S6**: *m/z* calcd. for $[M+6H]^{6+}$ 2686.1 (monoisotopic), found 2686.1.

OVA–Tn-Ser protected hexadecavalent oxime-linked construct 15. Glycodendrimer **S6** (2.84 mg, 0.18 µmol) was treated with TFA/CH_2Cl_2 (1:1, 0.5 mL) for 30 minutes at room temperature. The reaction mixture was added dropwise to ice-cold diethyl ether (5.0 mL), and the obtained solid was filtered, dried and used in the next step without further purification. Following general synthetic procedure G for disulfide bridging, the crude was dissolved in DMF/NaOAc buffer (pH 4.5, 40 mM) (2:1, 0.8 mL), and a solution of OVA peptide **10** (0.56 mg, 0.2 µmol, 1.1 equiv) in DMF (50 µL) was added. Protected construct **S15** (2.08 mg, 62% overall yield) was obtained after RP-HPLC purification (linear gradient 50–100% solvent B over 30 min) and lyophilization.

HPLC: t_R = 11.40 min (λ_{max} = 214 nm). **LRMS** (ESI$^+$-MS) *m/z*: Calcd. for $[M+7H]^{7+}$ 2670.8 (average), found 2668.3; calcd. for $[M+8H]^{8+}$ 2336.6, found 2336.0; calcd. for $[M+9H]^{9+}$ 2077.1, found 2077.4; calcd. for $[M+10H]^{10+}$ 1869.5, found 1869.7.

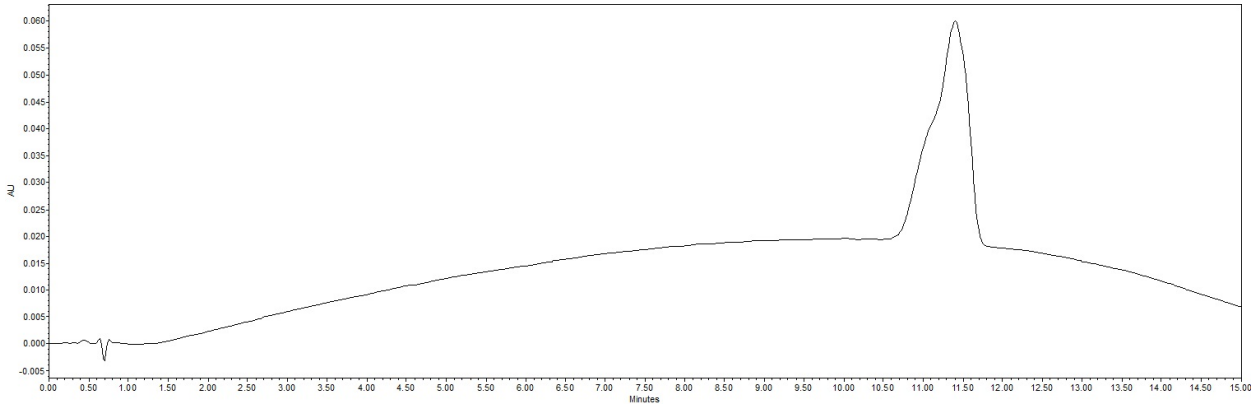


Fig. S27 Analytical RP-HPLC trace of **15**. t_R = 11.40 min (λ_{max} = 214 nm, 50–100% B over 15 min).

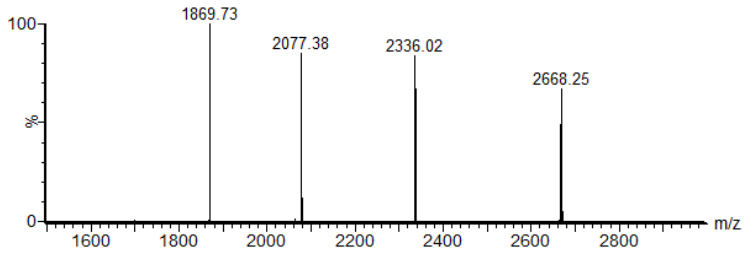
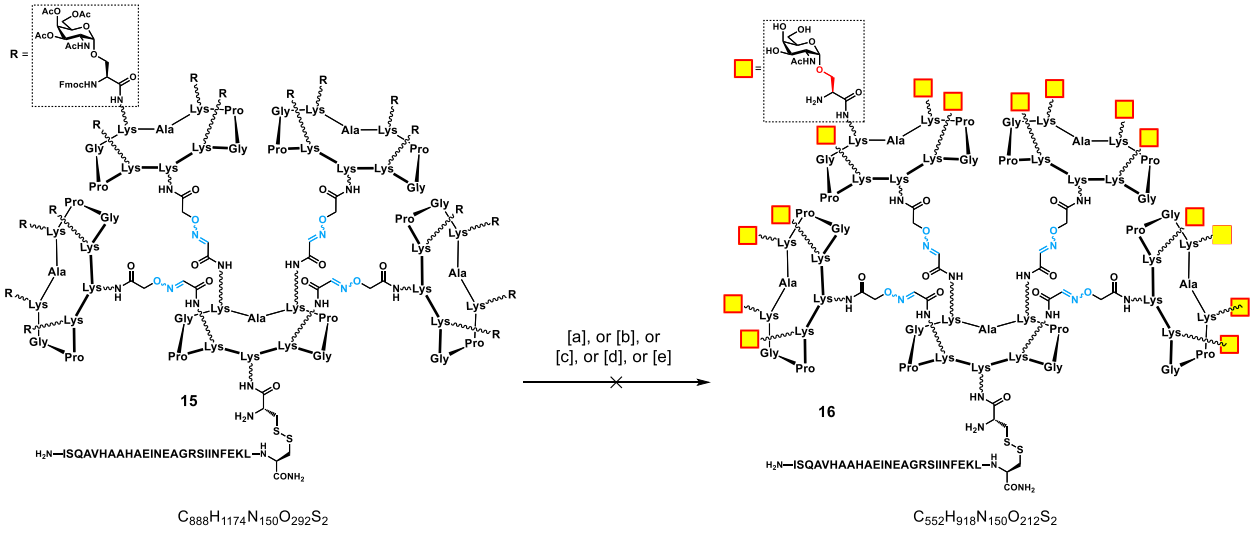
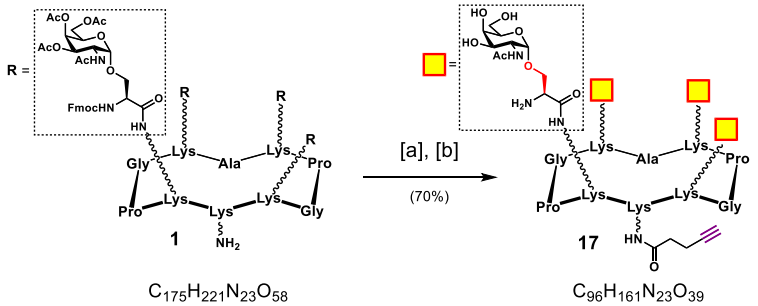


Fig. S28 LRMS (ESI$^+$-MS) spectrum of **15**: *m/z* calcd. for $[M+7H]^{7+}$ 2670.8 (average), found 2668.3; calcd. for $[M+8H]^{8+}$ 2336.6, found 2336.0; calcd. for $[M+9H]^{9+}$ 2077.1, found 2077.4; calcd. for $[M+10H]^{10+}$ 1869.5, found 1869.7.



Scheme S8 Global deprotection attempts toward final construct **16**. Reagents and conditions: [a] piperidine (32 eq.); [b] NaOMe/MeOH (pH≈10); [c] K_2CO_3 (20 mM); [d] 37% aq. HCl (50% v/v); [e] $MeHNNH_2$ (50% v/v). Reactions were carried out in dry MeOH (0.1-1.0 mM concentrations of **15**) at room temperature, progress was monitored via UPLC-MS at t=10, 30, 60, 120 min., to overnight.

Lys(4-Pentyn) Tn-Ser Tetravalent Glycodendrimer 18.



Scheme S9 Synthesis of glycodendrimer **17**. Reagents and conditions: [a] 4-pentynoic acid (1.5 equiv), DIPEA (2.0 equiv), PyBOP (2.0 equiv), DMF, r.t., 30 min; [b] $MeHNH_2$ (32 equiv), dry MeOH, r.t., overnight, 70% yield (over two steps).

To a solution of glycodendrimer **1** (104.2 mg, 29.1 µmol, 1.0 equiv) in DMF (5.0 mL), 4-pentynoic acid (4.3 mg, 43.8 µmol, 1.5 equiv), DIPEA (10 µL, 57.4 µmol, 2.0 equiv) and PyBOP (30.3 mg, 58.2 µmol, 2.0 equiv) were added. The reaction mixture was stirred for 30 min at room temperature, and then concentrated under high vacuum. The crude was dissolved in dry MeOH (2.0 mL), methylhydrazine (50.0 µL, 949.5 µmol) was added, and the reaction was stirred overnight at room temperature. The solvent was removed under reduced pressure and the crude was purified by RP-HPLC (linear gradient of 0–40% solvent B over 15 min) to give glycodendrimer **17** (46.1 mg, 70% yield) after lyophilization.

HPLC: t_R = 4.09 min (λ_{max} = 214 nm). **HRMS** (ESI$^+$-TOF) *m/z*: Calcd. for $[M+H]^+$ 2261.1400 (monoisotopic), found 2261.1311.

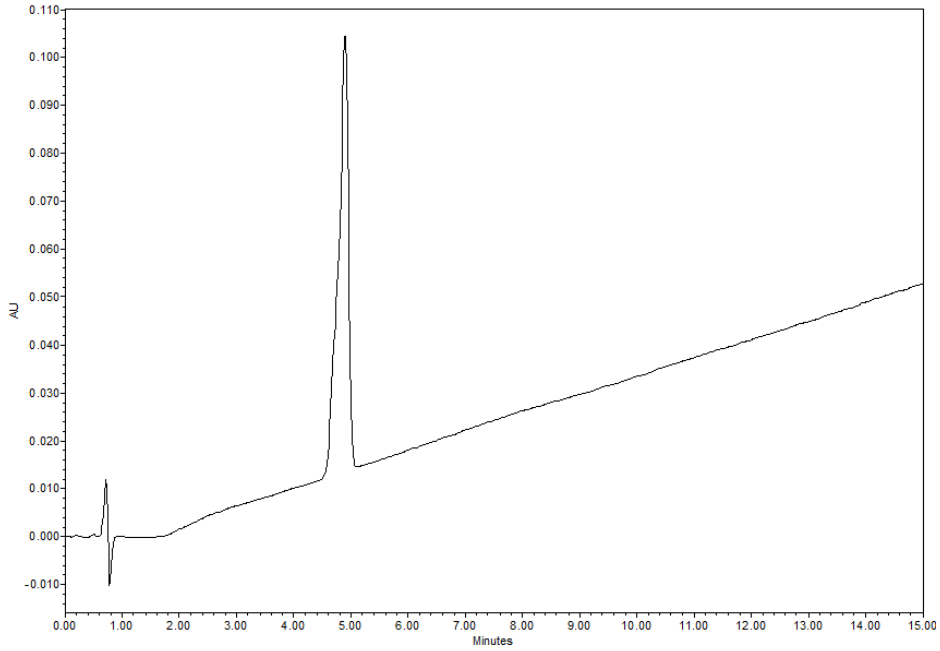


Fig. S29 Analytical RP-HPLC trace of **17**: t_R = 4.09 min (λ_{max} = 214 nm, 0–40% B over 15 min).

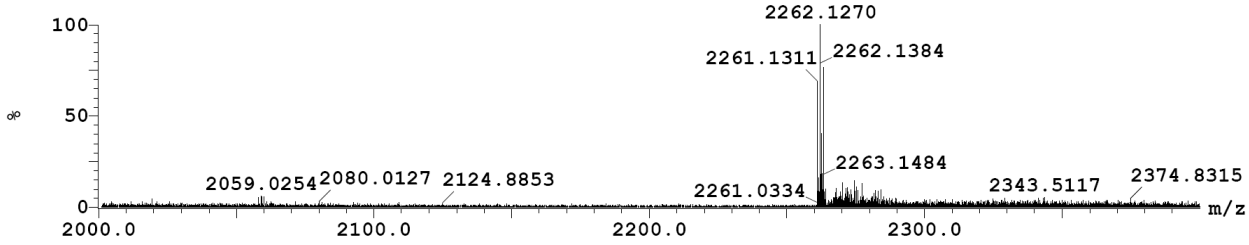
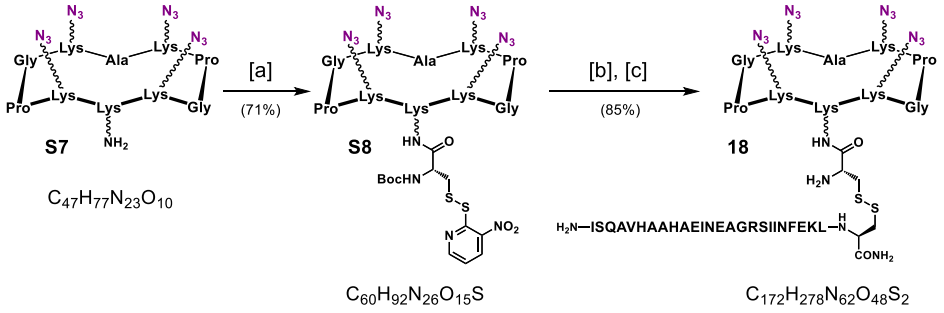


Fig. S30 HRMS (ESI$^+$-TOF) of **17**: m/z calcd. for $[M+H]^+$ 2261.1400 (monoisotopic), found 2261.1311.

OVA-functionalized Azide-bearing Tetravalent Scaffold 18.



Scheme S10 Synthesis of compound **18**. Reagents and conditions: [a] Boc-Cys(NPys)-NHS[6] (1.4 equiv), DIPEA (1.5 equiv), DMF, r.t., 30 min, 71% yield; [b] TFA/CH_2Cl_2 (1:1), r.t., 30 min; [c] peptide **10** (1.1 equiv), DMF/NaOAc buffer (pH 4.5, 40 mM) (2:1), r.t., 30 min, 85% yield (over two steps).

Boc-Cys(NPys) tetravalent scaffold S8. To a solution of **S7**[8] (16.2 mg, 14.4 µmol, 1.0 equiv) in DMF (2.0 mL), DIPEA (3.8 µL, 21.8 µmol, 1.5 equiv) and Boc-Cys(NPys)-NHS[6] (10.2 mg, 20.5 µmol, 1.4 equiv) were added. After stirring for 30 minutes at room temperature, the reaction mixture was directly purified by RP-HPLC (linear gradient of 5–100% solvent B over 30 min.) to give scaffold **S8** (15.1 mg, 71% yield) after lyophilization.

HPLC: t_R = 9.04 min (λ_{max} = 214 nm). **HRMS** (ESI$^+$-TOF) *m/z*: Calcd. for $[M+Na]^+$ 1503.6575 (monoisotopic), found 1503.6613.

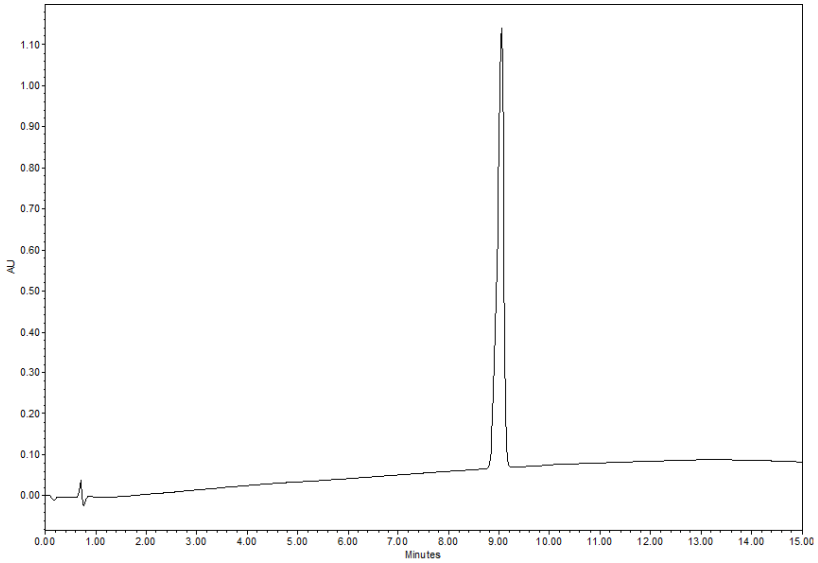


Fig. S31 Analytical RP-HPLC trace of **S8**. t_R = 9.04 min (λ_{max}= 214 nm, 5–100% B over 15 min).

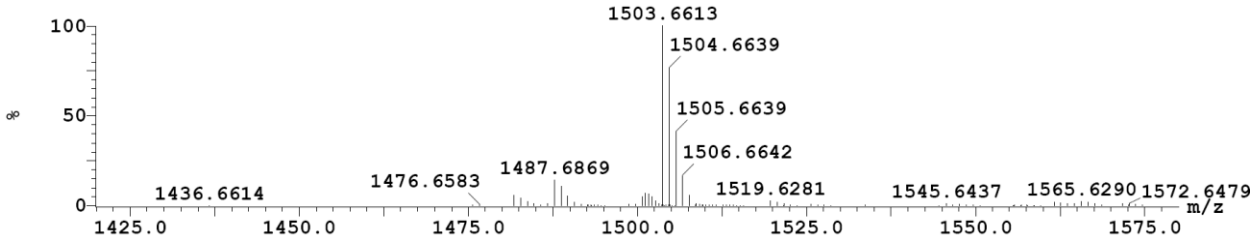


Fig. S32 HRMS (ESI$^+$-TOF) of **S8**: *m/z* calcd. for $[M+Na]^+$ 1503.6575 (monoisotopic), found 1503.6613.

OVA-functionalized azide-bearing tetravalent scaffold 18. Compound **S8** (6.2 mg, 4.2 µmol, X equiv) was treated with TFA/CH_2Cl_2 (1:1, 1 mL) for 30 minutes at room temperature. The reaction mixture was added dropwise to ice-cold diethyl ether (10 mL), and the obtained solid was filtered, dried and used in the next step without further purification. Following general synthetic procedure G for disulfide bridging, the crude was dissolved in DMF/NaOAc buffer (pH 4.5, 40 mM) (2:1, 0.8 mL), and a solution of OVA peptide **10** (13.0 mg, 4.6 µmol, 1.1 equiv) in DMF (0.2 mL) was added. Compound **18** (14.4 mg, 85% overall yield) was obtained after RP-HPLC purification (linear gradient 5–100% solvent B over 30 min) and lyophilization.

HPLC: t_R = 7.03 min (λ_{max} = 214 nm). **LRMS** (ESI$^+$-MS) *m/z*: Calcd. for $[M+2H]^{2+}$ 2024.3 (average), found 2024.5; calcd. for $[M+3H]^{3+}$ 1349.9, found 1350.3; calcd. for $[M+4H]^{4+}$ 1012.6, found 1012.6; calcd. for $[M+5H]^{5+}$ 810.3, found 810.3; calcd. for $[M+6H]^{6+}$ 675.4, found 675.4

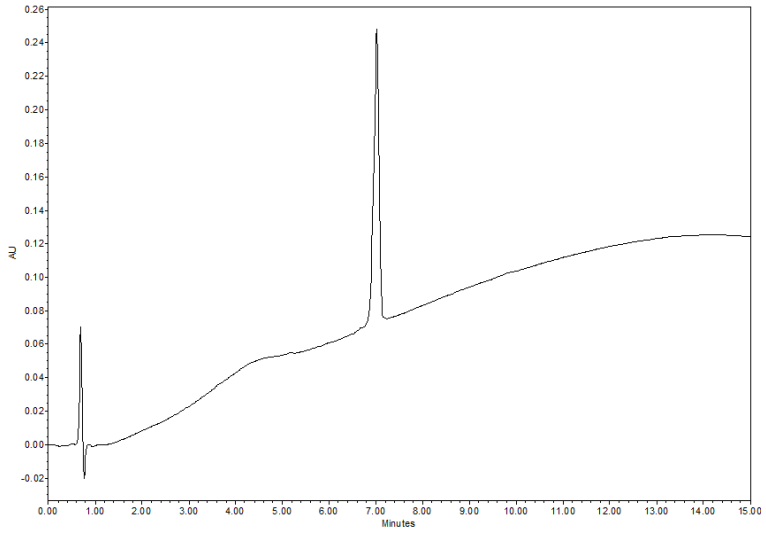


Fig. S33 Analytical RP-HPLC trace of **18**: t_R = 7.03 min (λ_{max} = 214 nm, 5–100% B over 15 min).

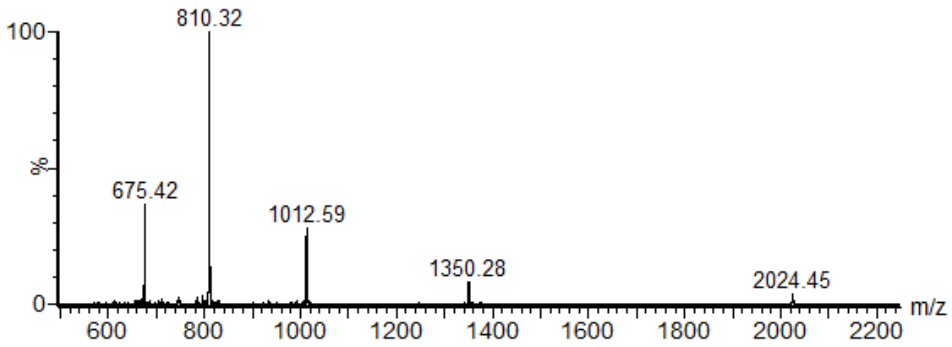
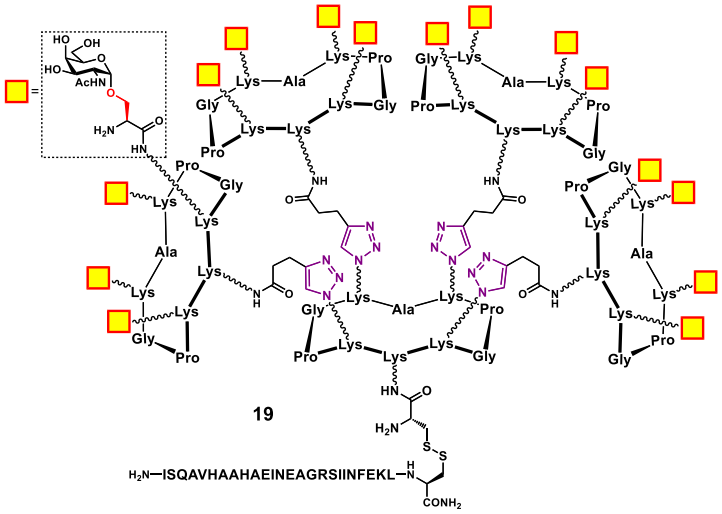


Fig. S34 LRMS (ESI$^+$-MS) spectrum of **18**: *m/z* calcd. for $[M+2H]^{2+}$ 2024.3 (average), found 2024.5; calcd. for $[M+3H]^{3+}$ 1349.9, found 1350.3; calcd. for $[M+4H]^{4+}$ 1012.6, found 1012.6; calcd. for $[M+5H]^{5+}$ 810.3, found 810.3; calcd. for $[M+6H]^{6+}$ 675.4, found 675.4.

OVA–Tn-Ser Hexadecavalent Triazole-linked Vaccine Construct 19.



$C_{556}H_{922}N_{154}O_{2042}S_2$

Following general synthetic procedure F for CuAAC, to compounds **18** (2.1 mg, 0.52 µmol, 1.0 equiv) and **17** (6.0 mg, 2.65 µmol, 5.0 equiv) dissolved in DMF (0.6 mL), a solution of THPTA (2.3 mg, 5.3 µmol, 10 equiv), $CuSO_4 \cdot 5H_2O$ (0.1 mg, 0.4 µmol, 0.8 equiv) and sodium ascorbate (3.1 mg, 15.6 µmol, 30 equiv) in aqueous PBS (pH 7.4, 10 mM, 1.2 mL) was added. Compound **19** (5.11 mg, 75% yield) was obtained after RP-HPLC purification (linear gradient 5–40% solvent B over 15 min) and lyophilization.

HPLC: t_R = 9.33 min (λ_{max} = 214 nm). **LRMS** (ESI$^+$-MS) *m/z*: Calcd. for $[M+5H]^{5+}$ 2619.5 (average), found 2617.5; calcd. for $[M+6H]^{6+}$ 2183.0, found 2182.7; calcd. for $[M+7H]^{7+}$ 1871.3, found 1871.6; calcd. for $[M+8H]^{8+}$ 1637.5, found 1638.0; calcd. for $[M+9H]^{9+}$ 1455.7, found 1456.1; calcd. for $[M+10H]^{10+}$ 1310.2, found 1310.4; calcd. for $[M+11H]^{11+}$ 1191.2, found 1191.1; calcd. for $[M+12H]^{12+}$ 1092.0, found 1092.1; calcd. for $[M+13H]^{13+}$ 1008.1, found 1008.3; calcd. for $[M+14H]^{14+}$ 936.2, found 935.9; calcd. for $[M+15H]^{15+}$ 873.8, found 874.1

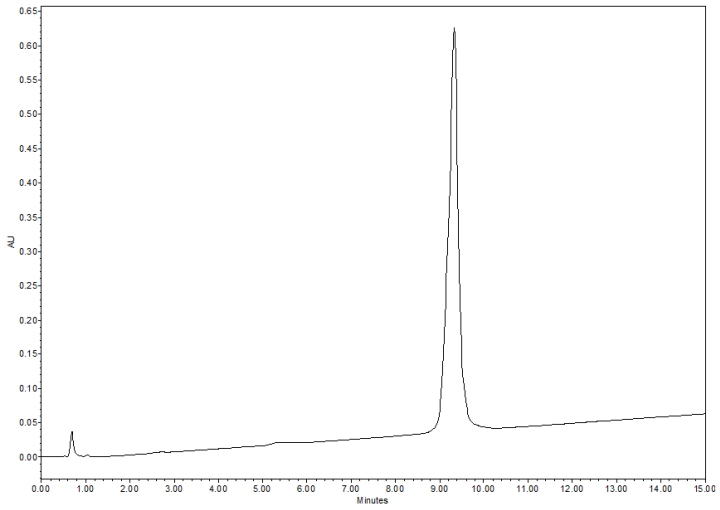


Fig. S35 Analytical RP-HPLC trace of **19**: t_R = 9.33 min (λ_{max} = 214 nm, 5–40% B over 15 min)

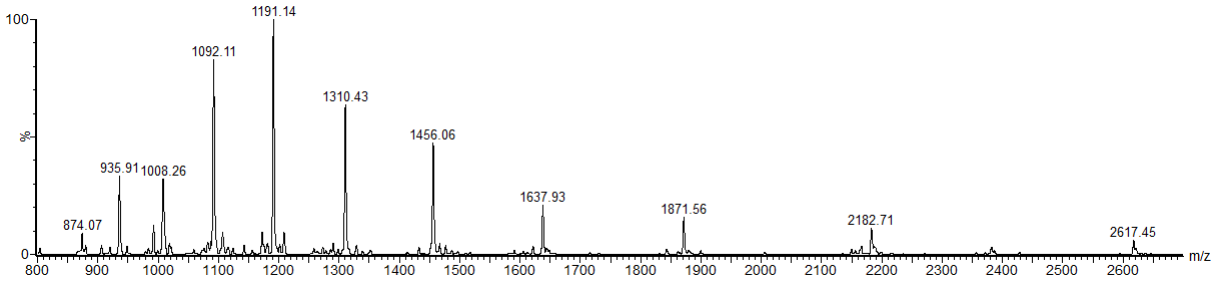
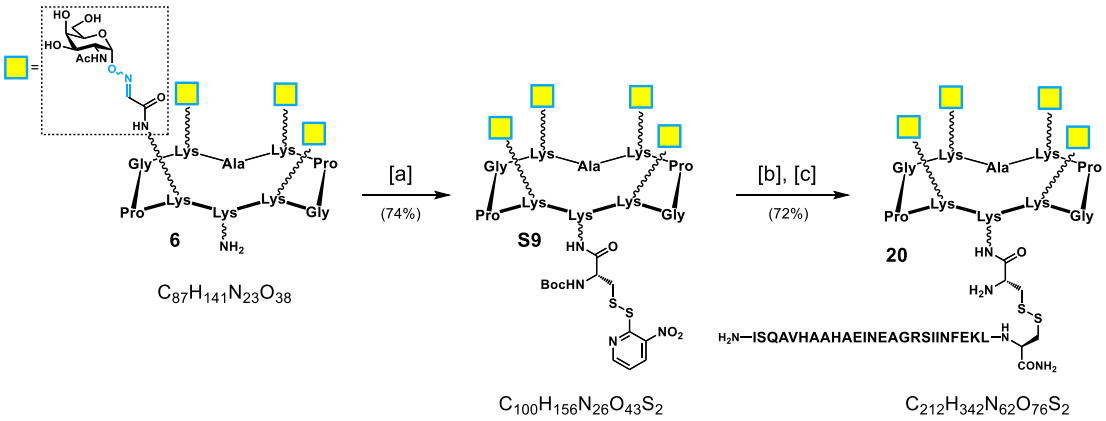


Fig. S36 LRSM (ESI$^+$-MS) spectrum of **19**: *m/z* calcd. for $[M+5H]^{5+}$ 2619.5 (average), found 2617.5; calcd. for $[M+6H]^{6+}$ 2183.0, found 2182.7; calcd. for $[M+7H]^{7+}$ 1871.3, found 1871.6; calcd. for $[M+8H]^{8+}$ 1637.5, found 1638.0; calcd. for $[M+9H]^{9+}$ 1455.7, found 1456.1; calcd. for $[M+10H]^{10+}$ 1310.2, found 1310.4; calcd. for $[M+11H]^{11+}$ 1191.2, found 1191.1; calcd. for $[M+12H]^{12+}$ 1092.0, found 1092.1; calcd. for $[M+13H]^{13+}$ 1008.1, found 1008.3; calcd. for $[M+14H]^{14+}$ 936.2, found 935.9; calcd. for $[M+15H]^{15+}$ 873.8, found 874.1.

B. Synthesis of Tn-oxime Constructs

OVA–Tn-oxime Tetravalent Construct 20.



Scheme S11 Synthesis of OVA–Tn-oxime tetravalent construct **20**. Reagents and conditions: [a] Boc-Cys(NPys)-NHS[6] (1.5 equiv), DIPEA (1.5 equiv), DMF, r.t., 30 min, 74% yield; [b] TFA/CH_2Cl_2 (1:1), r.t., 30 min; [c] peptide **10** (1.1 equiv), NaOAc buffer (pH 4.5, 40 mM), DMF, r.t., 20 min, 72% yield (over two steps).

Boc-Cys(NPys) Tn-oxime tetravalent glycodendrimer S9. To a solution of compound **6**[9] (14.0 mg, 6.6 µmol, 1.0 equiv) in DMF (1.5 mL), DIPEA (1.7 µL, 9.8 µmol, 1.5 equiv) and Boc-Cys(NPys)-NHS[6] (4.7 mg, 9.9 µmol, 1.5 equiv) were added. After stirring for 20 minutes, the reaction mixture was directly purified by RP-HPLC (linear gradient 5–60% solvent B over 30 min) and lyophilized to give Cys-functionalized glycodendrimer **S9** (12.0 mg, 74% yield).

HPLC: t_R = 8.15 min (λ_{max} = 214 nm). **HRMS** (ESI^+-TOF) *m/z*: Calcd. for $[M+H]^+$ 2475.0368 (most intense peak of isotopic cluster), found 2475.0386; calcd. for $[M+Na]^+$ 2496.0159 (monoisotopic), found 2496.0229.

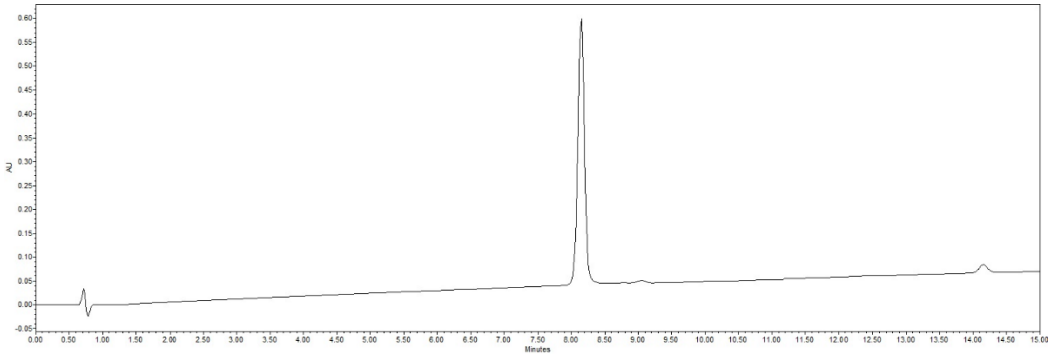


Fig. S37 Analytical RP-HPLC trace of **S9**: t_R = 8.15 min (λ_{max} = 214 nm, 5–60% B over 15 min).

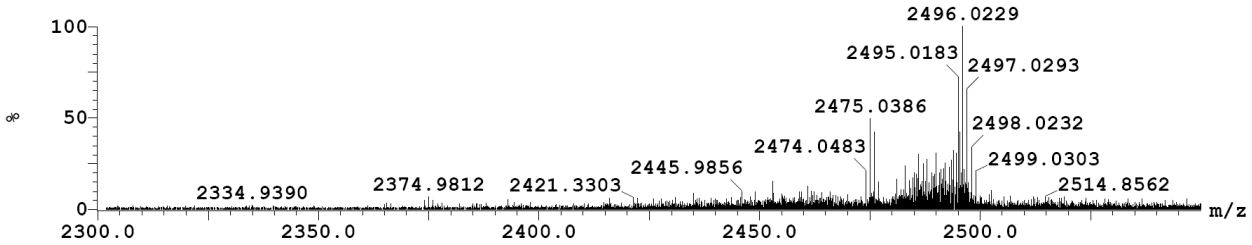


Fig. S38 HRMS (ESI$^+$-TOF) of **S9**: *m/z* calcd. for $[M+H]^+$ 2475.0368 (most intense peak of isotopic cluster), found 2475.0386; calcd. for $[M+Na]^+$ 2496.0159 (monoisotopic), found 2496.0229.

OVA–Tn-oxime Tetravalent Construct 20. Compound **S9** (8.4 mg, 3.4 µmol, 1.0 equiv) was treated with TFA/CH_2Cl_2 (1:1, 1.0 mL) for 30 min at room temperature. The reaction mixture was added dropwise to ice-cold diethyl ether (10 mL), and the obtained solid was filtered, dried and used in the next step without further purification. Following general synthetic procedure G for disulfide bridging, the crude was dissolved in NaOAc buffer (pH 4.5, 40 mM, 1.0 mL), and a solution of OVA peptide **10** (10.5 mg, 3.7 µmol, 1.1 equiv) in DMF (0.2 mL) was added. Compound **20** (12.3 mg, 72% overall yield) was obtained after RP-HPLC purification (linear gradient 0–40% solvent B over 30 min) and lyophilization.

HPLC: t_R = 11.97 min. (λ_{max} = 214 nm). **LRMS** (MALDI-TOF) *m/z*: Calcd. for $[M+H]^+$ 5040.5 (average), found 5041.4.

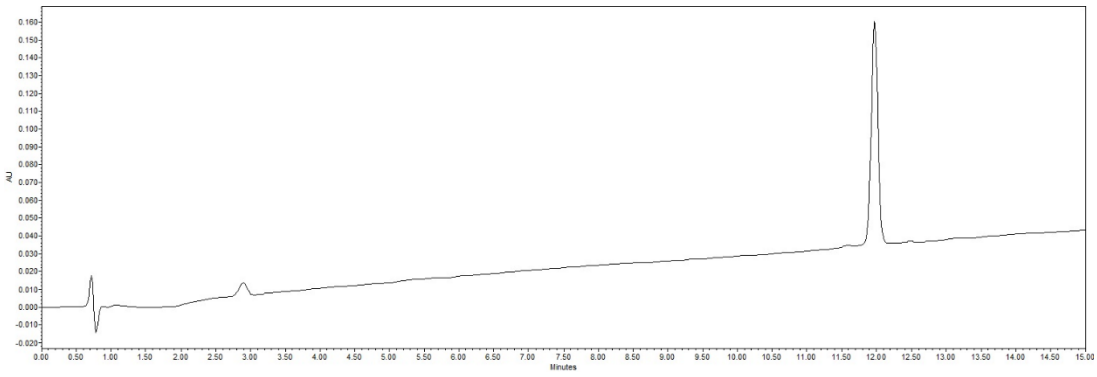


Fig. S39 Analytical RP-HPLC trace of **20**: t_R = 11.97 min (λ_{max} = 214 nm, 0–40% B over 15 min).

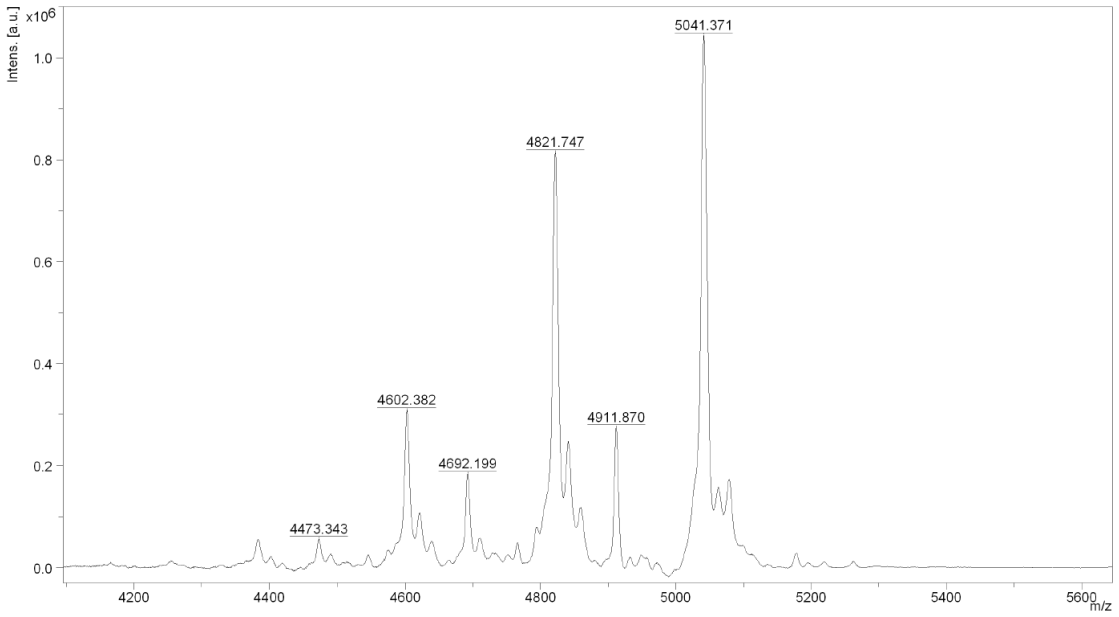
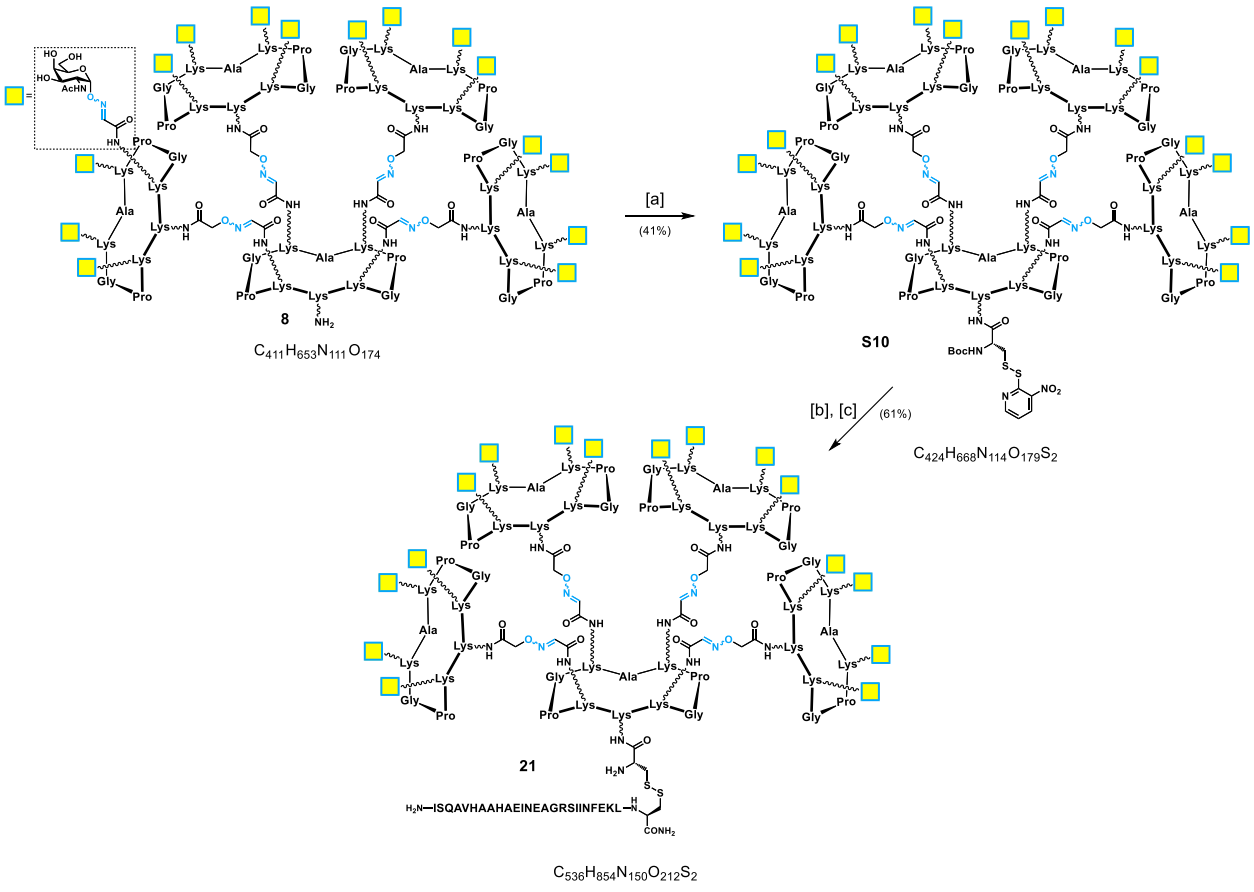


Fig. S40 LRMS (MALDI-TOF) spectrum of **20**: *m/z* calcd. for $[M+H]^+$ 5040.5 (average), found 5041.4.

OVA–Tn-oxime Hexadecavalent Vaccine Construct 21.



Scheme S12 Synthesis of Tn-oxime hexadecavalent vaccine construct 21/**23**. Ragents and conditions: [a] Boc-Cys(NPys)-NHS[6] (1.6 equiv), DMF/PBS (pH 7.4, 10 mM) (1:1), 1 h, 41% yield; [b] TFA/CH_2Cl_2 (1:1), r.t., 30 min; [c] **10** (1.2 equiv), NaOAc buffer (pH 4.5, 40 mM), DMF, r.t., 30 min, 61% yield (over two steps).

Boc-Cys(NPys) Tn-oxime hexadecavalent glycodendrimer S10. To a solution of glycodendrimer **8**[10] (7.9 mg, 0.8 µmol, 1.0 equiv) in a PBS(pH 7.4, 10 mM)/DMF mixture (1:1, 0.4 mL), Boc-Cys(NPys)-NHS[6] (0.6 mg, 1.3 µmol, 1.6 equiv) was added. After stirring for 1 h at room temperature, the reaction mixture was directly purified by RP-HPLC (linear gradient 0–40% solvent B over 30 min) and lyophilized to give Cys-functionalized hexadecavalent glycodendrimer **S10** (3.4 mg, 41% yield).

HPLC: t_R = 11.15 min (λ_{max} = 214 nm). LRMS (ESI$^+$-MS) m/z: Calcd. for $[M+4H]^{4+}$ 2573.7 (average), found 2574.1; calcd. for $[M+5H]^{5+}$ 2059.1, found 2060.5; calcd. for $[M+6H]^{6+}$ 1716.1, found 1717.0; calcd. for $[M+7H]^{7+}$ 1471.1, found 1472.1.

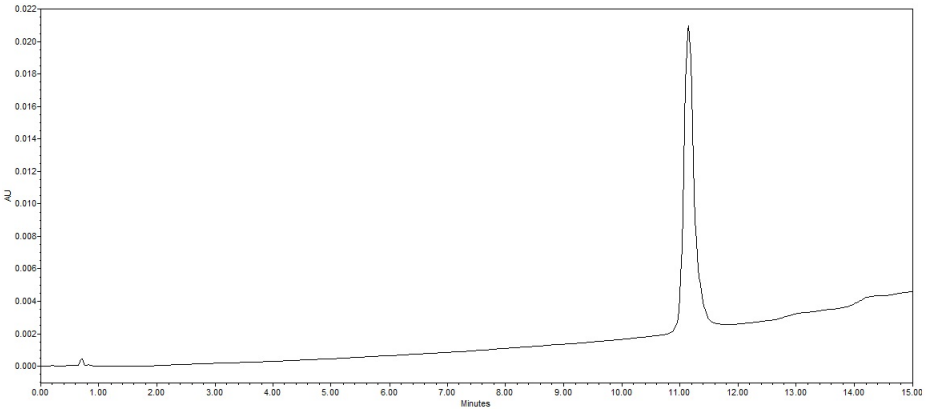


Fig. S41 Analytical RP-HPLC trace of **S10**: t_R = 11.15 min (λ_{max} = 214 nm, 0–40% B over 15 min).

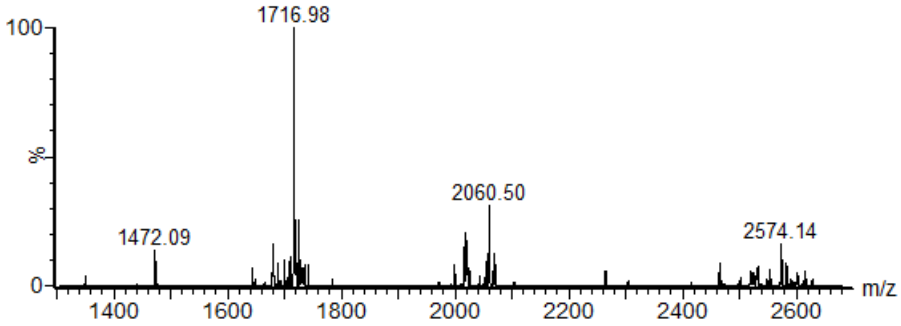


Fig. S42 LRMS (ESI$^+$-MS) spectrum of **S10**: *m/z* calcd. for $[M+4H]^{4+}$ 2573.7 (average), found 2574.1; calcd. for $[M+5H]^{5+}$ 2059.1, found 2060.5; calcd. for $[M+6H]^{6+}$ 1716.1, found 1717.0; calcd. for $[M+7H]^{7+}$ 1471.1, found 1472.1.

OVA–Tn-oxime Hexadecavalent Vaccine Construct 21. Compound **S10** (5.4 mg, 0.5 µmol, 1.0 equiv) was treated with TFA/CH_2Cl_2 (1:1, 1.0 mL) for 30 min at room temperature. The reaction mixture was added dropwise to ice-cold diethyl ether (10 mL), and the obtained solid was filtered, dried and used in the next step without any further purification. Following general synthetic procedure G for disulfide bridging, the crude was dissolved in NaOAc buffer (pH 4.5, 40 mM, 1.0 mL), and a solution of OVA peptide **10** (1.7 mg, 0.6 µmol, 1.2 equiv) in DMF (50 µL) was added. Compound **21** (3.9 mg, 61% overall yield) was obtained after RP-HPLC purification (linear gradient 5–60% solvent B over 30 min) and lyophilization

HPLC: t_R = 7.47 min (λ_{max} = 214 nm). **LRMS** (ESI$^+$-MS) *m/z*: Calcd. for $[M+5H]^{5+}$ 2572.1 (average), found 2572.3; calcd. for $[M+6H]^{6+}$ 2143.6, found 2144.1; calcd. for $[M+7H]^{7+}$ 1837.5, found 1838.0; calcd. for $[M+8H]^{8+}$ 1607.9, found 1608.4; calcd. for $[M+9H]^{9+}$ 1429.4, found 1429.9; calcd. for $[M+10H]^{10+}$ 1286.6, found 1287.0

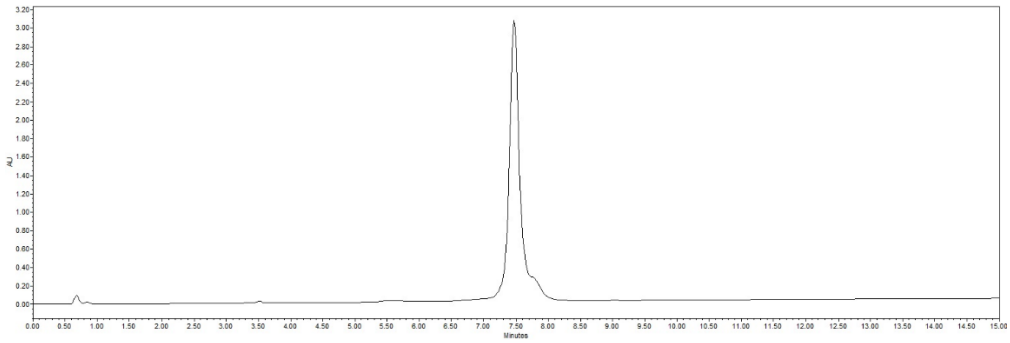


Fig. S43 Analytical RP-HPLC trace of 21/**23**: t_R = 7.47 min (λ_{max} = 214 nm, 5–60% B over 15 min).

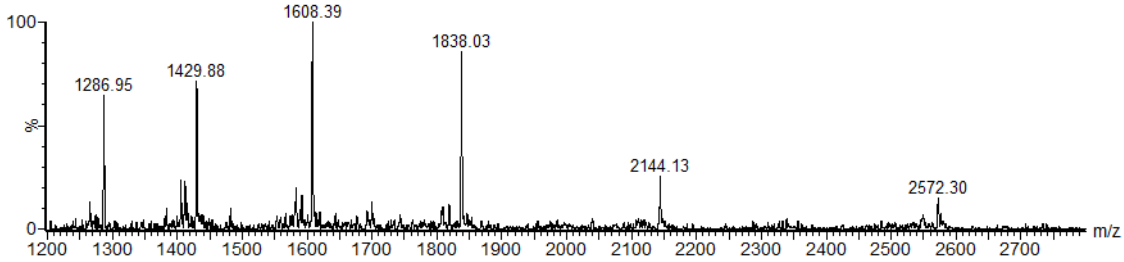


Fig. S44 LRMS (ESI$^+$-MS) spectrum of **21/23**: *m/z* calcd. for $[M+5H]^{5+}$ 2572.1 (average), found 2572.3; calcd. for $[M+6H]^{6+}$ 2143.6, found 2144.1; calcd. for $[M+7H]^{7+}$ 1837.5, found 1838.0; calcd. for $[M+8H]^{8+}$ 1607.9, found 1608.4; calcd. for $[M+9H]^{9+}$ 1429.4, found 1429.9; calcd. for $[M+10H]^{10+}$ 1286.6, found 1287.0.

IV. IMMUNOLOGICAL EVALUATION IN VITRO AND IN VIVO

A. DIRECT INTERACTION ELISA ASSAY WITH mAb 9A7

96-well microtiter Nunc-Immuno plates (Maxi-Sorp, Thermo Scientific) were coated with two-fold serial dilutions of each glycodendrimer (see Scheme 1: compounds **2**, **5**, **6**, **7** and **8**) in PBS buffer (pH 7.4, from 100 µM to 3 nM, 100 µL per well) for 1 h at 37 °C. The wells were then washed with T-PBS (PBS pH 7.4 containing 0.05% Tween 20, 3 × 100 µL per well). This washing procedure was repeated after each incubation step. The coated microtiter plates were then blocked with BSA in PBS (3% w/v, 100 µL per well) for 1 h at 37 °C. Primary mouse anti-Tn monoclonal antibody (9A7) was then added (100 µL per well) and plates were incubated for 1 h at 37 °C. Anti-Tn antibody interaction with coated glycodendrimers was revealed by using goat anti-mouse IgG peroxidase conjugate at a 1:1000 dilution for 1 h at 37 ºC (100 µL per well) and o-phenyldiamine (OPD)/H_2O_2 as a substrate (100 µL per well). The reaction was quenched after 10 min by adding H_2SO_4 (30% v/v, 50 µL per well) and the absorbance was measured at 490 nm. The optical density (OD) at 490 nm was plotted against the logarithm of the concentration for each glycodendrimer. Sigmoidal curves were fitted using Origin v6.1 software.

B. ANIMALS

Animals were cared for and handled in compliance with the Guidelines for Accommodation and Care of Animals (European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes) and internal guidelines. Mice were housed in ventilated cages and fed on a standard diet *ad libitum*. All the experimental procedures were approved by the appropriate local authorities. The CIC bioGUNE animal facility is fully accredited by AAALAC International.

C. MOUSE IMMUNIZATION

Groups of five mice (C57BL/6, female, 6-8 weeks old) were vaccinated subcutaneously three times every two weeks (days 0, 14, and 28) with the synthetic vaccine constructs (50 µg/mouse) co-administered with QS-21 as an adjuvant (20 µg/mouse, Desert King Int.) in phosphate-buffered saline (PBS, 10 mM, 100 µL/mouse). An additional group was immunized with the vaccine construct alone without adjuvant as a control. To assess antibody production, mice were bled by cardiac puncture at the experimental endpoint (day 50). Blood was collected in BD Microtainer® tubes (Clot Activator/SST™ Gel), allowed to clot over 2 h at 4 °C and centrifuged at 16 000*g* for 10 min. Sera were then harvested and stored at –20 °C until further analysis.

D. MEASUREMENT OF HUMORAL RESPONSE BY ELISA

Analysis of the produced antibodies in blood sera was carried out by an indirect enzyme-linked immunosorbent assay (ELISA). In brief, 96-well microtiter Nunc-Immuno plates (Maxi-Sorp,

Thermo Scientific) were coated with each of the glycodendrimers lacking the OVA epitopes (compounds **2**, **5**, **6** and **8**) at 5 µg/well for fixed sera dilution ELISAs and 0.5 µg/well in the titration assays (100 µL/well, PBS buffer (pH 7.4)). Plates were incubated overnight at 4 °C, washed four times with 10 mM PBS containing 0.05% Tween 20, and then blocked with 10% fetal bovine serum (FBS, Biowest) in PBS buffer (blocking buffer) for 1 h at room temperature. Diluted post-vaccination mouse sera (1/200 for total IgG analysis and serial dilutions for antibody titration) in blocking buffer were added to the wells of the coated plates and incubated for 1 h at room temperature. After wash, goat anti-mouse IgG (Jackson ImmunoResearch), as well as IgG1, IgG2b and IgG2c (Bio-Rad) or IgM (BD Pharmingen™) antibodies conjugated to horseradish peroxidase (HRP) were added to each well in blocking buffer at a 1/1000 dilution and plates were incubated for 1 h at room temperature. 3,3',5,5'-Tetramethylbenzidine (TMB) was added as a reaction substrate (100 µL/well of KPL SureBlue reserve™ commercial solution, SeraCare) and after incubation for 10 min, the reaction was stopped with 2 N H_2SO_4 (50 µL/well). Absorbance was immediately measured at 450 nm using a BioTek® Synergy HT multi-detection microplate reader. Initial total IgG analysis was presented as median OD values of five samples per group. Antibody titers were calculated as $\log_2$ values of the reciprocal endpoint dilution giving an OD higher than the selected cutoff, which was calculated following the method described by Frey *et al*, using the mean and the standard deviation of the pre-sera values.[11]

Analysis of IgM antibodies in blood sera. No IgM antibodies were found 49 days after first immunization, confirming the class switching towards IgG subclass.

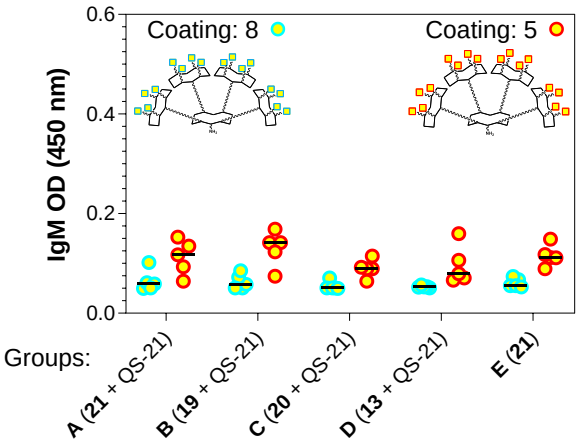


Fig. S45 IgM antibody production in blood sera 49 days after first immunization. Microtiter plates were coated with glycosylated scaffolds **8** or **5**. Horizontal bars (black) indicate median OD values of 5 mice per group.

E. Assessment of in vivo Cellular Response via T-cell Restimulation by Flow Cytometry

On day 50 after the first immunization, spleens were harvested and processed to obtain a primary splenocytes culture. Briefly, organs were smashed against a cell strainer (70 µm, Falcon™) and washed twice with cold washing buffer (1% FBS in PBS 10 mM). Red blood cell lysis was performed by resuspending the cell pellet in ACK lysing buffer (4 mL, 155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA) for 5 min at room temperature. After quenching with washing buffer, cells were counted and seeded in a 24-well plate ($3 \cdot 10^6$ cells/well) in TexMACS™ medium (1 mL/well, Miltenyi Biotec), either alone or in the presence of full length OVA protein as stimulus. After 48 h, cells were harvested and stained for further analysis of activation markers by flow cytometry. After wash, cells were blocked using Fc block™ antibody (anti-mouse CD16/CD32, BD Pharmingen™) at a 1/100 dilution in FACS buffer (1% BSA in PBS 10 mM) for 10 min at room temperature. Splenocytes were then stained using PE-Cy7-labelled anti-CD4, PerCP-Cy5.5-labelled anti-CD8, FITC-labelled anti-CD44 and PE-labelled anti-CD107a antibodies for 20 min at 4 °C in the absence of light. After extensive wash, cells were resuspended in FACS buffer and the expression of the different markers was assessed using a FACS Canto II flow cytometer. Data were analyzed using the FlowJo software (FlowJo, LCC). Cells were electronically gated based on the forward and side scatter parameters and the non-single events were left out based on forward area and height scatter parameters. The two different T cell populations were gated based on positive staining for CD4 and CD8 markers, respectively, and the expression of each activation marker was analyzed within this population.

F. Cell-Surface Reactivity of Antisera Against Tumor Cells by Immunofluorescence

MCF7 cell line was cultured using DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% of FBS (Gibco) and 1% of penicillin/streptomycin antibiotic solution (P/S, Gibco). For microscopy assays, MCF7 cells were collected by trypsinization (TrypLE Express, Gibco), seeded on top of sterile round cover glasses in 24-well plates ($2 \cdot 10^5$ cells/well) and allowed to adhere overnight. After washing with PBS (10 mM), cells were blocked with blocking buffer (10% FBS in PBS) for 1 h at 37 °C and then incubated for 2 h at 4 ºC with sera from immunized mice at a 1/100 dilution in blocking buffer (500 µL/well). Cells were extensively washed with PBS buffer, then FITC-labeled anti-mouse IgG secondary antibody (Sigma-Aldrich) diluted 1/32 in blocking buffer was added (500 µL/well) and cells were incubated for 2 h at 4 °C. Cells were washed again and fixed using a formaldehyde solution (3.7% in PBS, VWR) for 20 min at 4 °C. After this time, DAPI (Invitrogen) was added (5 µg/mL) to stain the nuclei and cells were incubated for 10 min at 4 °C. Finally, after extensive wash, glass coverslips were carefully mounted onto a microscope slide containing the ProLong™ Gold Antifade Mountant reagent (Invitrogen). Slides were kept at 4 °C in the darkness until further analysis. Appropriated controls were included, *i.e.* sera from non-

immunized mice, control for secondary antibody (no primary added) and positive control (commercial anti-Tn antibody, Invitrogen). An upright fluorescent microscope (Axio Imager D1, Carl Zeiss Microscopy, LLC) was employed to capture the images. Further image analysis was conducted using the ZEN 2.3 blue edition software.

G. STATISTICS

Antibody OD values and titers are presented as median of five mice per group. The statistical significance of the antibody response for each of the experimental groups compared to the 'no-adjuvant' control was assessed using a two-tailed unpaired Student's t-test with 95% confidence interval (CI),. *p* values equal to/less than 0.05 were considered statistically significant: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (GraphPad Prism, GraphPad Software, La Jolla, CA). Flow cytometry analysis data are presented as mean ± SEM of five mice.

V. ADDITIONAL REFERENCES

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