Synthesis of Clustered Glycoside – Antigen Conjugates by Two One-Pot, Orthogonal, Chemoselective Ligation Reactions: Scope and Limitations

Cyrille Grandjean,* Hélène Gras-Masse, and Oleg Melnyk*[a]

Abstract: Major histocompatibility class II antigens have been bound to clustered glycosides for selective targeting of the dendritic cell mannose receptor. Di-, tetra-, and octavalent glycoside – antigen conjugates have been obtained after two, orthogonal, hydrazone/thioether ligations, performed by using thio derivatives of D-mannose, D-galactose, or D(−)-quinic acid, glyoxylyl (or hydrazino)-*N*-chloroacetylated lysinyl trees, and N-terminal hydrazino (or glyoxylyl) peptide antigens. Successful one-pot condensations have been developed to account for the nature of the antigens and the valency of the trees.

Keywords: antigens · carbohydrates · chemoselectivity · dendrimers · hydrazones

Introduction

Selective targeting of dendritic cells promises much for synthetic vaccine strategies. Unique among leukocyte populations for their ability to initiate, stimulate, or inhibit the immune response,[1] dendritic cells are also particularly notable for their ability to activate helper-T cells, which in turn affect both cytotoxic T lymphocyte and humoral responses, by a mechanism involving presentation of exogenous antigen peptide fragments by the major histocompatibility class II molecules.[2] Presented antigens are internalized by the dendritic cells by fluid phase macropinocytosis or by an uptake mediated by a mannose receptor, then entering into the endocytic pathway for processing.[3] The latter mode of internalization has been reported by Engering et al.[4] and Tan et al.[5] to be between 200 and 10000 times more potent than endocytosis in in vitro stimulation of T cell clone proliferation. Thus, specific antigen delivery to the dendritic cells, via the mannose receptor, should overcome the intrinsic low immunogenicity of peptides and improve vaccine efficacy.

However, it has been reported that the mannose receptor can selectively bind and internalize molecules or microorganisms coated with D-mannose, L-fucose, or *N*-acetyl-D-glucosamine residues[6] by means of simultaneous interaction with several carbohydrate recognition domains, in accordance with the cluster effect.[7] Therefore, in the context of an ongoing vaccine program, a work plan was devised to synthesize antigens linked to numerous glycosides and to optimize antigen presentation by varying the nature, number, and interresidue separations of the glycosides and the antigens. Of the many natural or synthetic poly-mannosylated or -fucosylated mannose receptor ligands thus far reported,[8] only a few had been coupled to peptide antigens,[5] and these few, despite a heavy emphasis upon the essential role played by the mannose receptor, had been obtained by a strategy of a type seemingly inappropriate for the repetitive approach desirable for the preparation of a large set of ligands. Thus, the project comprised: a) the preparation of dendrimeric cores bearing two sets of chemocompatible functional groups, b) the synthesis of glycosides and antigens derivatized with complementary functional groups, and c) the subsequent assembly, after two orthogonal chemical ligations, of purified, fully deprotected partners (Scheme 1).

Chemical ligation, which consists of the chemoselective coupling of two unique and mutually reactive functional groups in aqueous media, has been developed largely in protein synthesis, scoring notable successes with both native chemical ligation and expressed protein ligation.[9] These methodologies have been extended to the preparation of peptide – oligonucleotide conjugates[10] and glycopeptides, and even to the remodeling of cell surfaces.[11] Still, the employment in biomolecular synthesis of multiple ligation is exceedingly rare. Various protein or peptide conjugates have been obtained using two sequential ligations of either oxime or thioether.[12] Transposition of native chemical ligation to solid-phase conditions enabled Canne et al.[13] and Camarero et al.[14] to prepare proteins and peptides by performing up to three ligations. Synthesis of template-assembled synthetic

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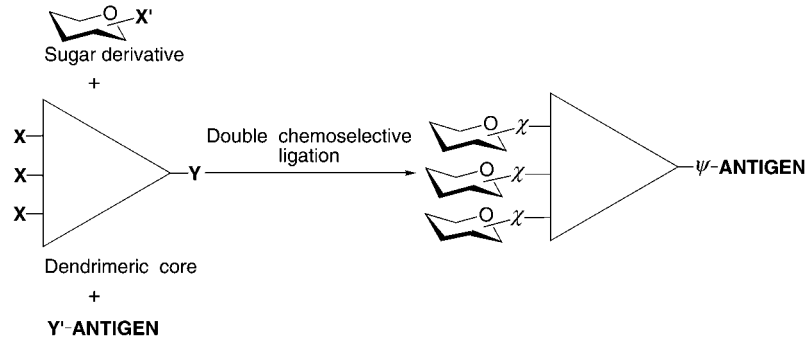


Scheme 1. Double ligation strategy for antigen-associated glycodendrimers. Present study: X = $ClCH_2CO-$; X′ = HS−; Y = NH_2–NH− (or HCOCO−); Y′ = HCOCO− (or NH_2–NH−); $\chi = -SCH_2CO-$; ψ = −NH–N= (or =N–NH−).

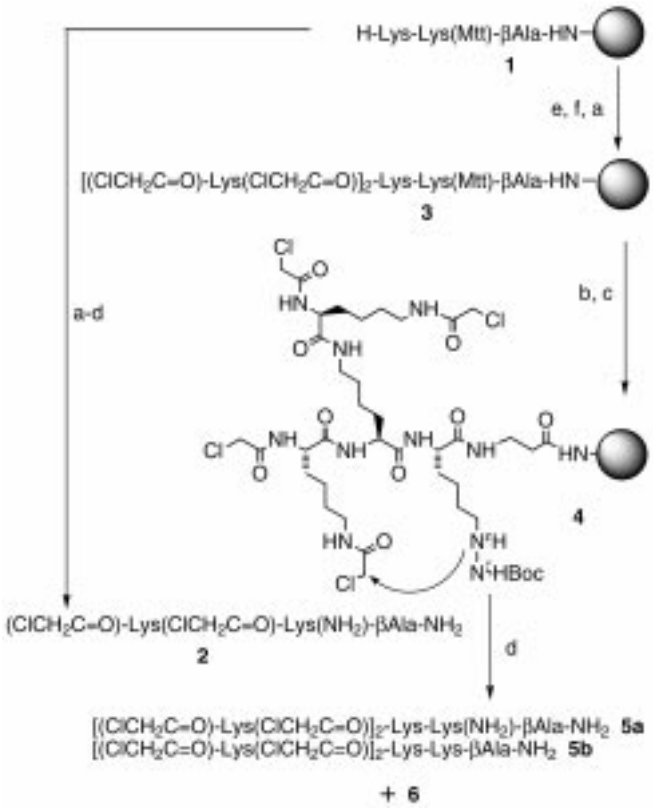
proteins (TASPs) provides further examples of stepwise oxime or thioether ligations.[15] With the exception of solid-phase chemical ligation, these syntheses required additional steps to unmask the reactive functional groups. To date, Canne's preparation of the cMyc-Max factor (a heterodimeric transcription factor) by means of thioester/oxime ligation,[16] remains the only example in which a biomolecule has been synthesized by a one-pot combination of two orthogonal ligations. Such a strategy has also been suggested by Mutter et al.[17] for TASP design.

We report here the synthesis of hydrazino (or glyoxylyl) *N*-chloroacetyl-L-lysinyl trees and their coupling with thio derivatives of D-mannose, D-galactose, or D-(−)-quinic acid, and *N*-terminal glyoxylyl (or α-hydrazinoacetyl) peptide antigens using novel, combined thioether/hydrazone ligation reactions (Scheme 1).

Results and Discussion

Ligation with hydrazino-*N*-chloroacetyl-L-lysinyl trees:

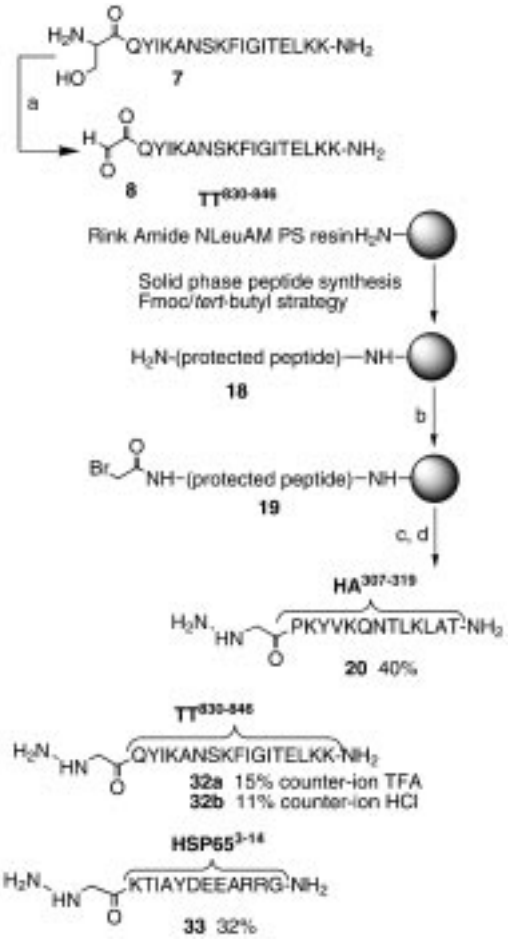
As a first approach, an investigation was undertaken of the reaction between *N*-chloroacetyl-L-lysinyl cores[18] bearing a hydrazine function, N-terminal glyoxylyl antigen peptides, and (2-thioethyl) α-D-mannopyranoside **10**. Di- and tetravalent chloroacetyl-lysinyl trees **2** and **5a** were elaborated by using the Fmoc/*tert*-butyl solid phase peptide synthesis strategy[19] on a Rink amide resin (Scheme 2). The ε-amino group of the first lysine to be introduced was deliberately not incorporated into the dendrimeric scaffold, so as to permit its later transformation into a hydrazino group by solid-phase N-electrophilic amination with *N*-Boc-3-(4-cyanophenyl)oxaziridine (BCPO).[20] The reaction between primary amines and BCPO is known for low yields, owing to the formation of Schiff bases between released 4-cyanobenzaldehyde and the unchanged amines. Adaptation of the N-electrophilic amination to the solid phase enabled the unwanted imines to be hydrolyzed selectively back to the starting amines, which could then be treated once more with BCPO. This cycle was repeated until



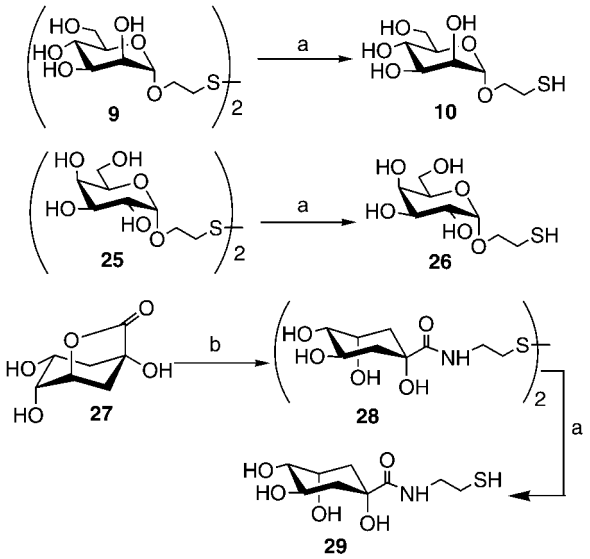
Scheme 2. Synthesis of hydrazino-(*N*-chloroacetyl)lysinyl cores. Compound **1** was synthesized on a Rink amide Nleu AM PS resin; a) $ClCH_2COOH$ (8 equiv/NH_2), DIC (4 equiv/NH_2), DMF, 1 h; b) TFA/CH_2Cl_2 1:99, continuous flow; c) BCPO (1 equiv), CH_2Cl_2, room temperature, 3 h, then *N*-benzylhydrazine dihydrochloride (1 equiv), DMF/AcOH/H_2O 85:15:5, 3 × 10 min, then neutralization with *i*$PrNEt_2$/CH_2Cl_2 5:95, 2 × 2 min, (all operations repeated 4 times); d) TFA/anisole 10:1, 0 °C, 1 h; e) Fmoc-L-Lys(Fmoc)-OH (4 equiv/NH_2), HBTU/HOBt/*i*$PrNEt_2$ (4:4:12 equiv/NH_2), NMP, 40 min; f) piperidine/NMP 20:80, 20 min; 20% overall yield for **2** and 8+4% overall yield for **5a** and **5b**, 3% for **6;** Boc = *tert*-butyloxycarbonyl; Mtt = 4-methyltrityl; DIC = diisopropylcarbodiimide; DMF = dimethylformamide; TFA = trifluoroacetic acid; Fmoc = 9-fluorenylmethoxycarbonyl; HBTU = *N*-[1*H*-benzotriazol-1-yl)-(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HOBt = 1-hydroxybenzotriazole; NMP = 1-methyl-2-pyrrolidone.

Abstract in French: *Des antigènes de classe II ont été liés à des clusters d'hydrates de carbone afin de permettre le ciblage du mannose récepteur des cellules dendritiques. Des antigènes conjugués à 2, 4 ou 8 glycosides ont été obtenus grâce à deux ligations orthogonales, hydrazone/thioéther, réalisées à partir de dérivés soufrés du* D-*mannose, du* D-*galactose ou de l'acide* D-*(−)-quinique, d'arbres de lysine N-chloroacétylés porteurs d'un groupe glyoxylyle (ou hydrazino) et d'antigènes modifiés par un groupe hydrazino (ou glyoxylyle) en position N-terminale. Des condensations en un seul pot efficaces ont pu être développées en fonction de la nature des antigènes et de la valence des arbres.*

complete transformation was achieved. The procedure was automated, and proved useful for the synthesis of various peptides containing Lys(NH_2), Orn(NH_2), or α-hydrazinoacetyl residues, which have already been employed successfully in hydrazone ligations.[21] Peptidyl resin **1** was first deprotected by treatment with 1% TFA in CH_2Cl_2,[22] and treated with BCPO (Scheme 2). After five cycles and cleavage from the resin, core **2** was obtained in 20% overall yield. The second generation core **5a** was secured in a much lower yield and proved to be contaminated by the unmodified, parent lysinyl tree **5b**(**5a**/**5b**: 2:1). Conversion was probably limited as a consequence of steric hindrance. In this case, the overall yield could not be improved by simply increasing the number of BCPO cycles: the electrophilic amination process in fact favored the formation of a more hydrophilic by-product (**6**), the NMR and mass spectral data of which were suggestive of a cyclic structure. Compound **6** arises from intramolecular substitution of a chlorine atom by the N^{ε}-end of the incoming hydrazine group during the neutralization step of the N-amination process.[23] Nonetheless, to validate the double ligation strategy, we synthesized the tetanus toxoid-derived, promiscuous immunogenic peptide $TT^{830-846}$, augmented with an *N*-terminal serine (**7**) (Scheme 3). Compound **7** was further oxidized to the α-oxo-aldehyde **8** in 72% yield, upon treatment with sodium metaperiodate.[24] (2-Thioethyl) α-D-mannopyranoside **10**[23] was selected as the glycoside partner. Stored as its disulfide derivative **9**, this compound was reduced by *n*-tributylphosphane prior to use (Scheme 4).



Scheme 3. Preparation of the antigens. a) $NaIO_4$ (1.2 equiv), AcOH/0.1M aq KH_2PO_4, room temperature, 5 min, then $HO(CH_2)_2OH$ in excess, 72%; b) bromoacetic acid (8 equiv), DIC (4 equiv), DMF, 20 min, then added to peptidyl resin, DMF, 1 h; c) $BocNHNH_2$ (4 equiv), *i*$PrNEt_2$ (6 equiv), DMF, overnight; d) TFA/anisole/H_2O 95:2.5:2.5, room temperature, 3 h, 40%.



Scheme 4. Preparation of the thio derivatives. a) *n*Bu_3P (1 equiv), *n*PrOH/H_2O 1:1, room temperature, overnight; b) $HS(CH_2)_2NH_2$, HCl (1 equiv), K_2CO_3, H_2O, 50 °C, overnight, then air, room temperature, 24 h; 71%.

Core **2** was first treated with α-oxo-aldehyde **8** in DMF/citrate-phosphate buffer (pH 5.2) (Scheme 5).[25] After completion of the reaction (monitored by RP-HPLC), **10** was introduced into the mixture and the pH was adjusted to 8–8.5 (paper) by addition of solid K_2CO_3[26–28] to give construct **11** in an isolated yield of 35% (Table 1, entry 1). A mixture of hydrazino and amino cores, **5a** and **5b**, was treated similarly to give the antigen-bearing cluster **12a**, together with the tetramannosylated L-lysinyl tree **12b**, in 37 and 52% yield, respectively (Figure 1, Table 1, entry 2). These encouraging results led to the proposition of a novel route, which would overcome the above-discussed difficulties associated with the preparation of trees functionalized with a hydrazino group, avoid the exposure of the antigen to sodium periodate, and be applicable to a broader range of epitopes. For example, the hydrazone ligation of peptides terminating in *N*-proline and modified with an α-glyoxylyl function is highly problematic. These derivatives in fact give rise, after nucleophilic intramolecular attack on the carbonyl group by the amide nitrogen of the third amino acid in the chain, to stable cyclic heterocycles.[29] This drawback might be circumvented by following a reverse strategy: that is, the coupling of glyoxylyl *N*-(chloroacetyl)lysinyl cores with hydrazino peptide antigens.

Ligation with glyoxylyl-*N*-chloroacetyl-L-lysinyl trees:

The lysinyl cores **15**, **16**, and **17** were synthesized in 18–31% yields on a poly(ethyleneglycol) dimethylacrylamide copolymer (PEGA) resin, using a novel linker[30] derived from tartaric acid, which generates the glyoxylyl group upon cleavage from the solid support.[31]

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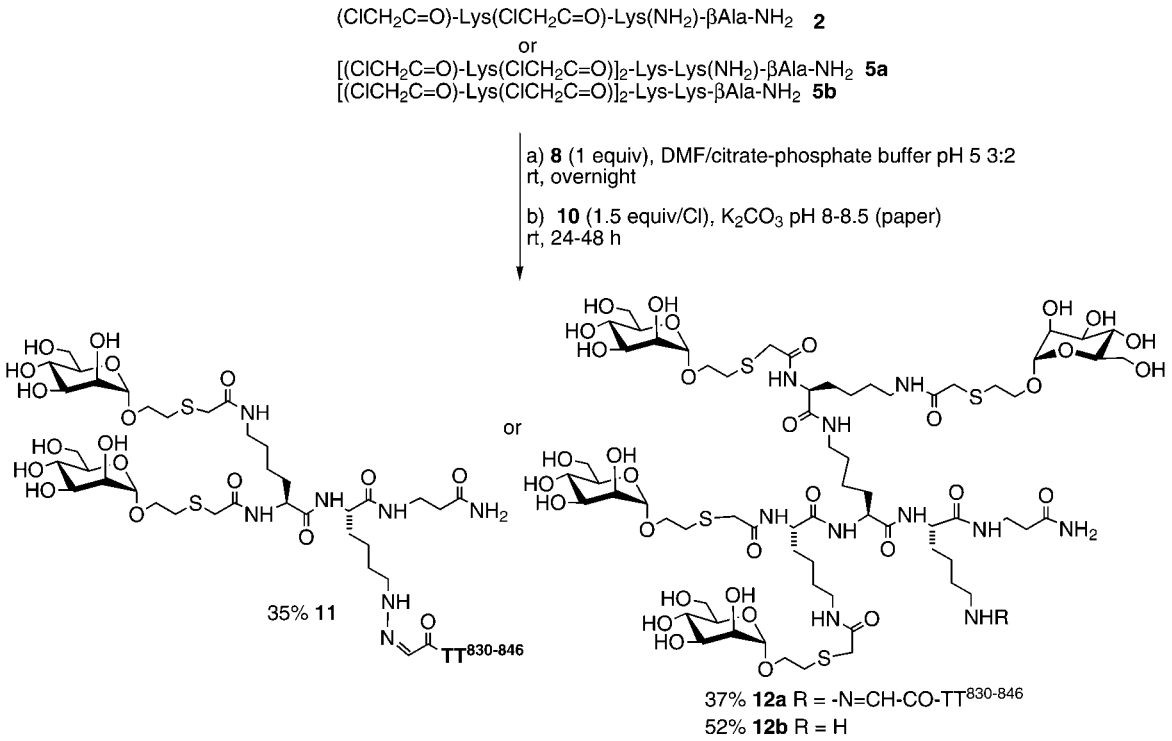


Scheme 5. Orthogonal hydrazone/thioether ligation reactions between hydrazino-(chloroacetyl)lysinyl cores, the glyoxylyl peptide antigen **8** and the thio sugar derivative **10**.

Table 1. Assembling of conjugates by double orthogonal ligation.

Entry	Thio compound	Tree	Antigen	Procedure[a]	Comments or yield [%]
1	**10**	**2**	**8**	A	35
2	**10**	**5a**	**8**	A	37
3	**10**	**15**	**20**	A	43
4	**10**	**16**	**20**	A	40
5	**10**	**17**	**20**	A	18
6	**10**	**17**	**20**	B	51
7	**26**	**17**	**20**	B	41
8	**29**	**17**	**20**	B	35
9	**10**	**17**	**33**	B	45
10	**10**	**17**	**32a**	B	aggregation
11	**10**	**16**	**32a**	B	aggregation
12	**10**	**16**	**32a**	[b]	aggregation
13	**10**	**16**	**32a**	[c]	40% conversion[d]
14	**10**	**16**	**32a**	C	59
15	**26**	**16**	**32a**	C	42
16	**29**	**16**	**32a**	C	35

[a] Procedure A: hydrazone ligation in DMF/citrate-phosphate buffer pH 5 3:2, then thioether ligation; procedure B: thioether ligation in DMF/H_2O 90:10, K_2CO_3, then hydrazone ligation in DMF/citrate – phosphate buffer; procedure C: hydrazone ligation in 2-methyl-2-propanol/H_2O 90:10, then thioether ligation. [b] Thioether/hydrazone DMF/0.1M NaOAc pH 4.6. [c] Thioether/hydrazone DMF/H_2O/*t*BuOH 4:1:5. [d] Estimated yield from RP-HPLC.

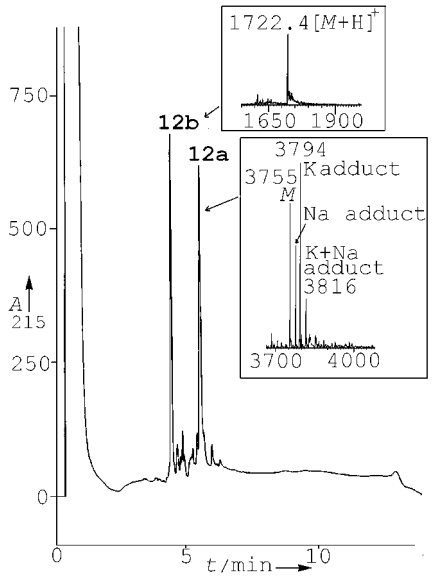
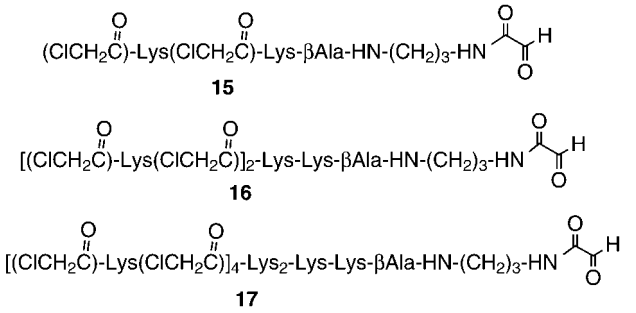


Figure 1. RP-HPLC chromatogram of the crude mixture from the sequential hydrazone/thioether ligation of epitope **8**, mixture of trees **5a**/**5b** and sugar derivative **10**. Chromatographic conditions: TSK gel (Toso-Haas) C18 (110 Å, 2 μm, 4.6 × 50 mm). Flow rate 1.5 mL min^{-1}, 50 °C. Buffer A: 0.05% aqueous TFA. Buffer B: 0.05% TFA in CH_3CN/H_2O 80/20. Gradient: 0% B for 5 min, 0 – 100% B over 10 min, 100% B for 1 min, 0% B for 2 min; the upper inset and the lower inset correspond to the positive ESI-MS of compound **12b** and to ESI-MS true mass scale of compound **12a**, respectively.

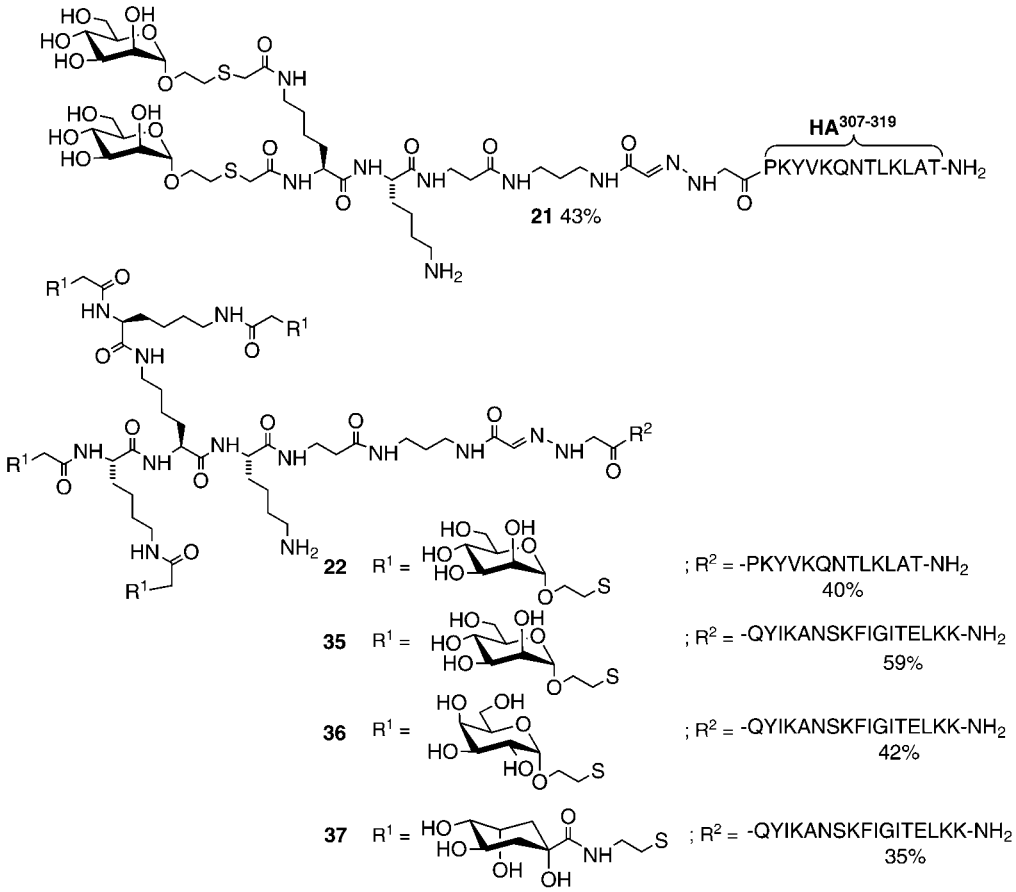
The synthesis of an *N*-proline-terminated epitope HA$^{307–319}$ was undertaken in parallel (Scheme 3). Modification of the hydrazino moiety was achieved by acylation, with bromoacetic acid anhydride, of the peptidyl resin **18** at its N-terminus to give **19**, followed by nucleophilic substitution with commercial *tert*-butylcarbazate.[32]

Modified antigen **20** was finally obtained, as the sole product and in 40% overall yield, after deprotection and cleavage from the resin by treatment with acid. It proved necessary to adopt the Fmoc/*tert*-butyl synthesis strategy and not to use acetonitrile as an RP-HPLC purification solvent.[33]



With these building blocks in hand, the next step was to examine their ability to convert into the target molecules. A one-pot double orthogonal reaction was used. Compounds **15**, **16**, and **17** were coupled with hydrazino antigen **20** to furnish the corresponding ligated fragments (as detected by RP-HPLC). Then, the second ligation was accomplished by addition of **10** and adjustment of the pH to 8–8.5, to give constructs **21**, **22**, and **24** in 43, 40, and 18% yields, respectively (Schemes 6 and 7, Table 1, entries 3–5). However, these buffer conditions were detrimental to the thioetherification, which was very sluggish for the preparation of octavalent conjugate **24**, as reflected in the actual yield and the complex RP-HPLC profile (Figure 2a, b). As explained previously,[26, 34] the presence of water reduces the nucleophilicity of the thiol, thus lowering the reaction rate and leading to incomplete substitution, while simultaneously favoring the disulfide formation that consumes the starting material. Thus, we considered performing the thioetherification before the hydrazone ligation in almost dry DMF, since the glyoxylyl group, being stable, would not be expected to interfere during the substitution reaction. The thioetherification proceeded smoothly to furnish intermediate **23** (Figure 2c), which was further treated with **10**. The presence in the crude mixture of the desired tree **24**, together with reformed disulfide **9**, was confirmed by RP-HPLC, in marked contrast with the profile observed when the alternative route was applied (compare Figure 2d and b). We were actually able to obtain the fully assembled octameric construct **24** in a considerably improved yield of 51% (Table 1, entry 6).[35]

The same conditions were applied to the ligation of antigen **20**, lysinyl core **17**, and 2-thioethyl α-D-galactopyranoside **26**[23] and the thio derivative of D-(−)-quinic acid **29**. The latter compound was obtained in 71% yield from quinide **27**,[36] which was treated with cysteamine in degassed H_2O. Compound **29** was purified and stored as its disulfide derivative **28**, and reduced prior to use (Scheme 4). Quinic acid was chosen since, apart from its use in evaluating the scope of the methodology, it can act as a potential mannopyranose mimic to the mannose receptor,[37] whereas galactose should provide conjugates unrecognizable, a priori, by this receptor. Octameric constructs **30** and **31** were obtained by



Scheme 6. Products of orthogonal hydrazone/thioether ligation reactions between glyoxylyl-(chloroacetyl)lysinyl cores **15** and **16**, the hydrazino peptide antigens, and the thio sugar derivatives.

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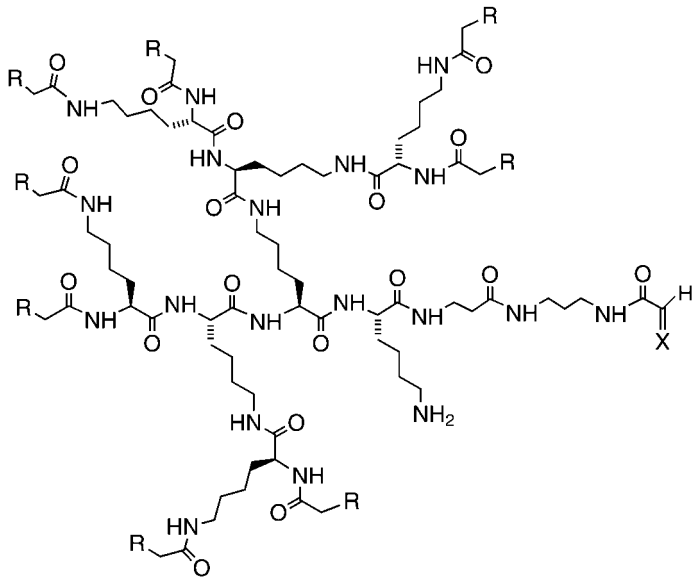
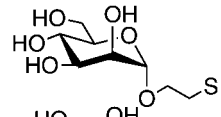
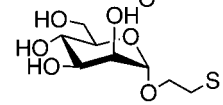
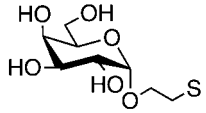
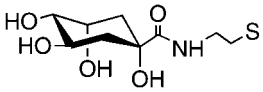
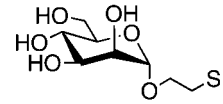


23 R =  ; X = O

24 R =  ; X = N-GPKYVKQNTLKLAT-NH_2
Hydrazone/thioether ligation 18%
Thioether/hydrazone ligation 51%

30 R =  ; X = N-GPKYVKQNTLKLAT-NH_2
41%

31 R =  ; X = N-GPKYVKQNTLKLAT-NH_2
35%

34 R =  ; X = N-GKTIAYDEEARRG-NH_2
45%

Scheme 7. Products of orthogonal hydrazone/thioether ligation reactions between glyoxylyl(chloroacetyl)lysinyl core **17**, the thio sugar derivatives and the hydrazino peptide antigens.

following the sequential thioether/hydrazone procedure, in 41 and 35% isolated yield, respectively (Table 1, entries 7 and 8).

The next study was of variation in the nature of the antigen. Thus, compounds **32a** and **33** (epitopes $TT^{830-846}$ and $HSP65^{3-14}$, modified at their N-termini by an α-hydrazinoacetyl group) were synthesized as described for compound **20** and obtained in 15 and 32% overall yields (Scheme 3). Although the hydrazone ligation step was slower than observed previously, **33** reacted under mild conditions with lysinyl core **17** and mannose derivative **10** to furnish the double-ligated construct **34** (Table 1, entry 9) in 45% yield (60% based on recovered epitope) (Scheme 7). However, **32a** led to a complex mixture when treated under the same conditions. Tetanus toxin derivative **32a** proved to be stable in a DMF/citrate-phosphate buffer for at least two hours (Figure 3, entry a), but aggregated in a few minutes upon coupling either with tree **17** (Figure 3b, Table 1, entry 10) or with glycosylated trees. The same reaction profile was observed by analytical RP-HPLC when the reaction was conducted with the smaller tree-like compound **16** (Table 1, entry 11), or when switched from citrate – phosphate to the occasional substitute,[25b–d, 25g] 0.1 M acetate buffer (pH 4.6), (Table 1, entry 12). To enhance the hydrophilic character of the difficult epitopic sequence for **32a**, trifluoroacetate counterions were substituted with chloride moieties to give **32b**. No improvement in the reaction profile was observed, however. The influence of the solvent was then examined. The use of DMSO, known to disrupt aggregation of peptides and to increase the hydrazone ligation rate considerably (compared to DMF)[25f] was precluded by the thioether ligation. As an alternative, we had found that 2-methyl-propan-2-ol had a beneficial effect upon hydrazone ligations that involved hydrophobic partners such as lipopeptides.[38] And indeed, performance of the hydrazone ligation in a *t*BuOH/DMF/citrate – phosphate buffer reduced aggregation and permitted partial coupling (Table 1, entry 13). The condensation of epitope **32a** with core **16** in *t*BuOH/H_2O 90/10 (i.e., in the absence of DMF and salts) was attempted next (Table 1, entry 14). Hydrazone ligation went to completion overnight under these conditions, although the material proved not entirely soluble (Figure 3c and d). The crude mixture was treated further with 2-thioethyl α-D-mannopyranoside **10** dissolved in H_2O, in the presence of K_2CO_3 at room temperature, to give a clean polysubstitution (Figure 3e). Tetramannosylated epitope conjugate **35** was obtained in 59% yield after RP-HPLC purification (Scheme 6). The double ligation was not as efficient when performed using a reverse procedure; that is, thioether followed by hydrazone condensation. Nor was it when the experiments were repeated with epitope **32b**, with

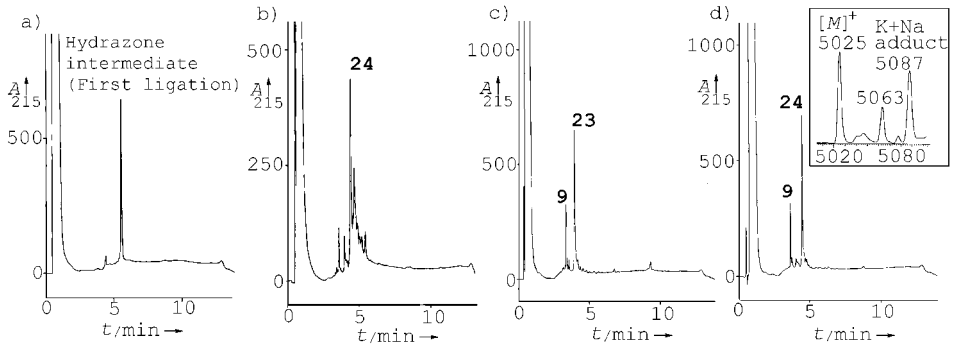


Figure 2. Synthesis and characterization of octavalent compound **24**. RP-HPLC chromatogram of the crude mixtures from: a) the hydrazone ligation of tree **17** and hydrazino antigen **20;** b) the thioether ligation of the hydrazone intermediate with sugar derivative **10;** c) the thioether ligation of tree **17** with 2-thioethyl α-D-mannopyranoside **10** after 36 h; d) the second ligation with hydrazinoantigen **20** overnight; chromatographic conditions: TSK gel (TosoHaas) C18 (110 Å, 2 μm, 4.6 × 50 mm). Flow rate 1.5 mL min^{-1}, 50 °C. Buffer A: 0.05% aqueous TFA. Buffer B: 0.05% TFA in CH_3CN/H_2O 80/20. Gradient: 0% B for 5 min, 0–100% B over 10 min, 100% B for 1 min, 0% B for 2 min; the inset corresponds to the ESI-MS true mass scale of compound **24**.

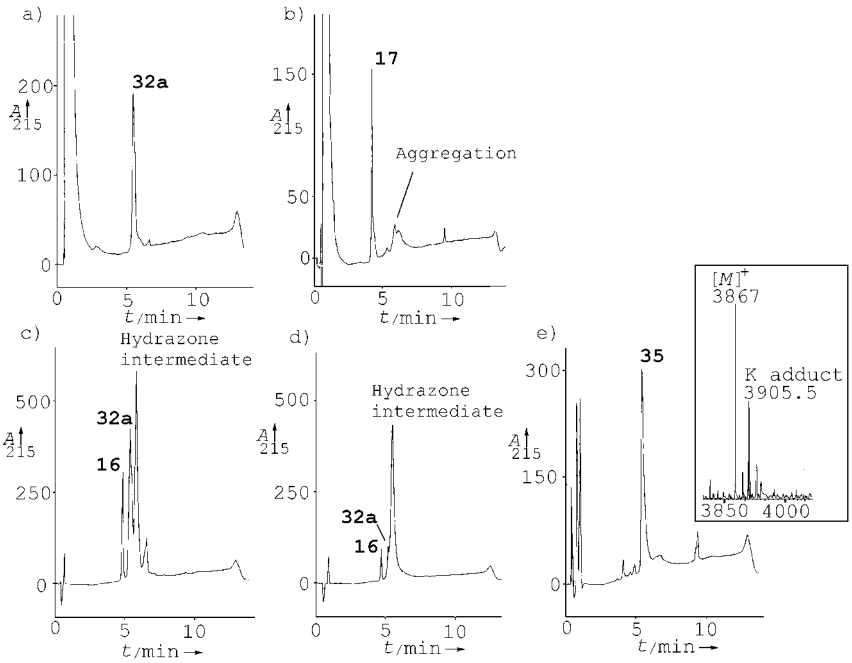


Figure 3. Stability of epitope **32a** and synthesis and characterization of tetravalent construct **35**. RP-HPLC chromatograms from: a) and b) epitope **32a** in DMF/citrate–phosphate buffer (pH 5) (3:2, room temperature), 2 h alone and in the presence of tree **17**, 10 min, respectively; c) and d) hydrazone ligation of tree **16**, epitope **32a** in *t*BuOH/H_2O 90/10, after 1 h and overnight, respectively; chromatographic conditions: TSK gel (TosoHaas) C18 (110 Å, 2 μm, 4.6 × 50 mm). Flow rate 1.5 mL min^{-1}, 50 °C. Buffer A: 0.05% aqueous TFA. Buffer B: 0.05% TFA in CH_3CN/H_2O 80/20. Gradient: 0% B for 5 min, 0–100% B over 10 min, 100% B for 1 min, 0% B for 2 min; the inset corresponds to the ESI-MS true mass scale of compound **35**.

chloride counter-ions. Finally, conjugates **36** and **37** (Table 1, entries 15 and 16) were assembled, following the hydrazone/thioether sequence in *t*BuOH, from core **16**, epitope **32a**, and either compound **26** or **29**, in 42 and 35% yield, respectively (Scheme 6).

Conclusion

This study provides new insights into chemoselective ligation. In particular, the reliability of combined thioether/hydrazone ligation for the preparation of conjugates has been demonstrated. *N*-chloroacetylated lysinyl trees, modified with either a hydrazino or a glyoxylyl group, proved to be valuable intermediates. Moreover, the latter are very promising since they are readily accessible and bear the two sets of electrophilic groups, thus preventing side reactions. In the context of our studies, the sequential procedure, using buffered DMF or 2-methyl-propan-2-ol, or employing the reversed order of ligation reactions, facilitated the preparation of antigens linked to up to four glycosides. To date, no limitation has been found regarding the thio-functionalized segment. This procedure can also circumvent the difficulties related to the coupling of epitopes terminating in *N*-proline. Further studies will be required to extend this methodology to the preparation of octameric trees linked to difficult epitopic sequences. However, vectorization of antigens by tetrameric glycoside clusters seems to be sufficient for targeting the mannose receptor.[5a]

Experimental Section

General: Analytical reverse-phase high performance liquid chromatography (RP-HPLC); separations were performed either upon a Beckman System Gold or on a Shimadzu LC-9A system, using a TSK super ODS gel (TosoHaas) (110 Å, 2 μm, 4.6 × 50 mm) column at a flow rate of 1.5 mL min^{-1} (monitoring and analysis), a Vydac C18 (100 Å, 2 μm, 4.6 × 300 mm) or a Beckman Ultrapore C18 (100 Å, 5 μm, 4.6 × 250 mm) at a flow rate of 1 mL min^{-1} (analysis), with detection at 215 or 230 nm at 50 °C. Semipreparative RP-HPLC separations were performed on a Shimadzu LC-4A system, on a Hypersil hyperprep C-18 (300 Å, 8 μm, 10 × 260 mm) column at a flow rate of 3 or 5 mL min^{-1}, with detection at either 215 or 230 nm at 50 °C. Solvent system A: 0.05% TFA in water; solvent system B: 0.05% TFA in 80% acetonitrile/20% water; solvent system C: 0.05% TFA in 60% acetonitrile/40% water; solvent system D: 0.03% HCl in water; solvent system E: 0.03% HCl in 80% acetonitrile/20% water; solvent system F: 0.05% TFA in 40% *i*PrOH/60% water; solvent system G: 0.03% HCl in 40% *i*PrOH/60% water. ESI-MS spectra were recorded on a Micromass Quatro II Electrospray Mass Spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX 300 spectrometer in H_2O/D_2O 90:10. Chemical shifts are given in ppm and referenced to internal 3-trimethylsilyl-[D_4-2,2,3,3]propionic acid sodium salt (TMSP).

Compounds were checked for homogeneity by analytical RP-HPLC, using two different solvent systems (solvent systems A/B and A/F), and by analytical capillary zone electrophoresis in a 75 μm × 500 mm fused silica capillary, with a 28 mA current and a 30 kV field in an Applied Biosystems Model 270A-HT system (Foster City, USA). Separations were performed either at 50 °C using a 50 mM sodium borate migration buffer at pH 9.2, or a 20 mM citrate buffer at pH 2.47 and at 40 °C. Lysinyl trees and epitopes were also characterized by amino acid analysis after total acidic hydrolysis of the peptidyl resin with 1 mL of propionic acid/12 N HCl (1:1) at 110 °C for 24 h.

Synthesis of 2-{N^{α},N^{ε}-di-[(1S_n,3*R*,4S_n,5*R*)-1,3,4,5-tetrahydroxycyclohexane-1-carboxamido-1-yl]}ethyl disulfide 28: A mixture of quinic acid 1,5-lactone **27** (52 mg, 0.3 mmol) and cysteamine hydrochloride (34 mg, 1 equiv) in degassed H_2O containing solid K_2CO_3 (pH 8–9, paper) was stirred overnight at 50 °C. The mixture was further stirred at room temperature in the presence of air for another 24 h before being purified by RP-HPLC [gradient: 100–0 to 80–20 (A/C), 15 min, then isocratic] to furnish **28** (70 mg, 71%) as a white powder after lyophilization: ^{1}H NMR: δ = 8.24 (t, ^3J(H,H) = 5.8 Hz, 2H; 2 NH), 4.05 (ddd, ^3J(H,H) = 3, 3, and 6.2 Hz, 2H; 2 H-3), 3.90 (ddd, ^3J(H,H) = 4.7, 9, and 11.6 Hz, 2H; 2 H-5), 3.40 (t, ^3J(H,H) = 6.4 Hz, 4H; 2 CH_2), 3.38 (dd, ^3J(H,H) = 3 and 9 Hz, 2H; 2 H-4), 2.73 (t, ^3J(H,H) = 6.4 Hz, 4H; 2 CH_2), 1.94–1.83 (m, 6H; 4 H-2 and 2 H-6), 1.77 (dd, ^3J(H,H) = 11.6 and 13.4 Hz, 2H; 2 H-6′); ^{13}C NMR: δ = 177.5 (2 CON), 77.8 (2 C-1), 75.4 (2 C-4), 70.8 (2 C-3), 66.7 (2 C-5), 40.7 (2 C-6), 38.5, 37.5, and 37.1 (4 CH_2 and 2 C-2); TOF-PDMS: *m/z*: 469 $[M+H]^+$.

Solid-phase peptide synthesis: All peptidic compounds were synthesized using the Fmoc/*tert*-butyl strategy. The coupling steps were performed using a fourfold excess of amino acid per amine subjected to HBTU/HOBt/DIEA activation in NMP.

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Synthesis of the hydrazino-*N*-chloroacetylated lysinyl trees: These compounds were synthesized manually on a Rink amide-Nleu-AM-PS resin (substitution, 0.38 mmol g^{-1}) (Senn Chemicals), by using the Fmoc/*tert*-butyl strategy. Lysinyl core of valency 2 as synthesized on a 0.25 mmol scale. Lysinyl core of valency 4 was synthesized on a 0.1 mmol scale. Typically, with coupling monitored by the TNBS test,[39] HBTU dissolved in NMP (4 equiv) was added to a mixture of the amino acid (4 equiv), HOBt (4 equiv), and DIEA (8 equiv) in NMP. After stirring for 1 min, the mixture was added to the peptidyl resin (1 equiv) solvated in NMP containing DIEA (4 equiv), and mechanically shaken for 40 min. After filtration, the peptidyl resin was washed with NMP (3×2 min), and CH_2Cl_2 (3×2 min). Each coupling was followed by a capping with Ac_2O/DIEA/CH_2Cl_2 10/5/85 for 10 min, followed by washing with CH_2Cl_2 (3×1 min). Cleavage of the Fmoc protecting groups was achieved by treatment with 20% piperidine in NMP (1×2 min, 1×20 min), followed by washing first with NMP (2×2 min), and then with CH_2Cl_2 (2×1 min). Fmoc-β-Ala-OH was anchored to the resin first (for the preparation of the second generation lysinyl cores, 0.26 equiv of Fmoc-β-Ala-OH was coupled, to diminish the load to 0.1 mmol). Chain protection of the first lysinyl residue introduced was effected by means of a 4-methyltrityl moiety. The other lysinyl residues were added as their Fmoc-L-Lys(Fmoc)-OH derivatives. After the third and fourth coupling steps, the peptidyl resin was deprotected and acylated using a fourfold excess of preformed chloroacetic anhydride, prepared by a DCC activation, to provide the first and second level carrier cores. The methyltrityl group of the ε-NH_2 of the first lysine residues was removed by a continuous flow of 1% TFA in CH_2Cl_2. The three cores were then subjected to the N-electrophilic amination procedure. The peptidyl resins were placed in an Applied Biosystems 431A peptide synthesizer (Foster City, CA), and subjected five times to the following cycles: 1) *N*-Boc-3-(4-cyanophenyl)oxaziridine (Acros Organics, Noisy le Grand, France) (1 equiv) in CH_2Cl_2 (6 mL) for 30 min (Boc-3-(4-cyanophenyl)oxaziridine was introduced as a solid in the cartridges normally used for peptide synthesis. The reagent was dissolved in the cartridge with CH_2Cl_2 and the solution transferred immediately to the reaction vessel); 2) CH_2Cl_2 and DMF washes; 3) *N*-benzylhydrazine dihydrochloride (50 mg) in DMF/Ac_2O/H_2O 85/15/10 (5 mL) for 30 min, 3 times; 4) DMF and CH_2Cl_2 washes; 5) 5% DIEA in CH_2Cl_2 for 2 min, twice; 6) CH_2Cl_2 washes. A Kaiser test was performed after these cycles to monitor conversion. The cores were then cleaved from the resin and deprotected by TFA/anisole 10/1 (11 mL per gram of peptidyl resin), for 1 h at 0 °C, precipitated in cold diethyl ether/heptane 1/1, centrifuged, dissolved in water and lyophilized.

Compound 2 (26 mg, 20%) was obtained as a white powder after RP-HPLC purification [gradient: 100:0 to 90:10 (A/B), 20 min], followed by lyophilization; ^{1}H NMR: $\delta = 8.37$ (d, $^3J(H,H) = 6.3$ Hz, 1H; lysyl $^{\alpha}$NH), 8.28 (d, $^3J(H,H) = 7.1$ Hz, 1H; lysyl $^{\alpha}$NH), 8.14 (br t, 1H; lysyl $^{\varepsilon}$NH), 8.00 (t, $^3J(H,H) = 5.7$ Hz, 1H; alanyl NH), 7.41 and 6.71 (2 br s, 2×1H; NH_2), 4.15 – 4.06 (m, 2H; $2 \times$ lysyl α-H), 4.00 and 3.95 (2 s, 2×3H; $2 \times CH_2$), 3.38 – 3.27 (m, 2H; β-alanyl β-H), 3.10 (dt, $^3J(H,H) = 6.6$ and 6.6 Hz, 2H; lysyl ε-H), 2.98 (t, $^3J(H,H) = 7.6$ Hz, 2H; lysyl ε-H), 2.33 (t, $^3J(H,H) = 6.4$ Hz, β-alanyl α-H), 1.67 – 1.46 (m, 6H, $4 \times$ lysyl β-H and $2 \times$ lysyl δ-H), 1.43 – 1.30 (m, 2H; $2 \times$ lysyl δ-H),1.29 – 1.17 (m, 4H; $4 \times$ lysyl γ-H); ^{13}C NMR: $\delta = 177.1$, 174.2, 173.9, 170.2, and 170.0 (5 NC=O), 54.6 and 54.0 ($2 \times$ lysyl α-C), 51.5 (lysyl ε-C), 39.8 (lysyl ε-C), 36.2 and 34.9 (β-alanyl α-C and β-C), 42.7 and 42.4 ($2 \times CH_2$), 30.8 and 30.7 ($2 \times$ lysyl β-C) 28.0 (lysyl δ-C), 24.0 (lysyl δ-C), 22.5 and 22.4 ($2 \times$ lysyl γ-C); TOF-PDMS: *m/z*: 576 $[M+(CH_3)_2C+Na]^+$, 554 $[M+(CH_3)_2C]^+$.

Compound 5a (25 mg, 12%) was obtained as a white powder (contaminated (ca. 33%) with the unreacted lysinyl core) after RP-HPLC purification [gradient: 100:0 to 80:20 (A/B), 20 min, then 80:20 to 70:30 (A/B), 25 min], followed by lyophilization together with a more hydrophilic cyclic by-product, **6** (6 mg, 3%), formed by intramolecular nucleophilic substitution of one chloride by the NH$^{\varepsilon}$ of the hydrazino group (the ability of this by-product to react with α-oxo-aldehyde to furnish hydrazone was used to confirm the integrity of the NH$^{\varepsilon}$); for **5a** and **5b**: positive ESI-MS: *m/z*: 922 $[M+H]^+$, 480 $[M+H+K]^{2+}$ and 907 $[M+H]^+$, 473 $[M+H+K]^{2+}$, respectively. for **6** (3%), ^{1}H NMR: $\delta = 8.34$ (d, $^3J(H,H) = 7.2$ Hz, 1H; lysyl $^{\alpha}$NH), 8.29 (d, $^3J(H,H) = 6.4$ Hz, 1H; lysyl $^{\alpha}$NH), 8.28 (d, $^3J(H,H) = 8.2$ Hz, 1H; lysyl $^{\alpha}$NH), 8.19 (d, $^3J(H,H) = 8.4$ Hz, 1H; lysyl $^{\alpha}$NH), 8.10 (t, $^3J(H,H) = 5.9$ Hz, 1H; lysyl $^{\varepsilon}$NH), 8.03 (t, $^3J(H,H) = 6.0$ Hz, 1H; lysyl $^{\varepsilon}$NH), 8.00 (t, $^3J(H,H) = 5.9$ Hz, 1H; lysyl $^{\varepsilon}$NH), 7.91 (t, $^3J(H,H) = 6.0$ Hz, 1H; β-alanyl NH), 6.71 and 7.42 ($2 \times$ br s, $2 \times$ H; NH_2), 4.24 – 4.01 (m, 4H; $4 \times$ lysyl α-H), 3.99, 3.97, and 3.94 ($3 \times$ s, 3×2H; $3 \times -COCH_2Cl$), 3.52 and 3.47 (2 AB systems, $^3J(H,H) = 14.2$, 2×1H; $-COCH_2NH-$), 3.37 – 3.17 (m, 2H; β-alanyl β-H), 3.11 – 2.97 (m, 6H, $6 \times$ lysyl ε-H), 2.70 – 2.90 (m, 2H; $2 \times$ lysyl ε-H), 2.33 (t, $^3J(H,H) = 6.7$ Hz, 2H; β-alanyl α-H), 1.68 – 1.52 (m, 8H, $8 \times$ lysyl β-H), 1.42 – 1.27 (m, 8H, $8 \times$ lysyl δ-H), 1.25 – 1.13 (m, 8H, $8 \times$ lysyl γ-H); positive ESI-MS: *m/z*: 886 $[M+H]^+$, 462 $[M+H+K]^{2+}$.

Synthesis of the glyoxylyl-*N*-chloroacetylated lysinyl trees: Synthesis and full characterization of these constructs will be reported elsewhere.[31]

Synthesis of the antigen peptides: All epitopes were synthesized on a 0.25 mmol scale on a Rink amide-Nleu-AM-PS resin (substitution, 0.38 mmol g^{-1}) (Senn Chemicals), on an Applied Biosystems A431 peptide synthesizer. The side chain protecting group for Fmoc-L-Lys-OH was *tert*-butoxycarbonyl; the side chain protecting group for Fmoc-L-Ser-OH, Fmoc-L-Glu-OH, Fmoc-L-Thr-OH, Fmoc-L-Tyr-OH, and Fmoc-L-Asp-OH was a *tert*-butyl group; the side chain protecting group for Fmoc-L-Asn-OH, Fmoc-L-Gln-OH, and Fmoc-L-His-OH was a trityl group; the side chain protecting group for Fmoc-L-Arg-OH was a 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl group.

HCO-CO-TT$^{830-846}$ (8): The peptide was cleaved from the resin and deprotected using TFA/H_2O/iPr_3SiH 95/2.5/2.5 (10 mL per gram of peptidyl resin), for 3 h at room temperature, precipitated in cold diethyl ether, centrifuged, dissolved in water and lyophilized. Peptide Ser-TT$^{830-846}$ (**7**) (38.5 mg, 6%) was obtained as a white powder after semi-preparative RP-HPLC [gradient: 100:0 to 75:25 (A/B), 50 min] purification and lyophilization; TOF-PDMS: *m/z*: 2068 $[M+H]^+$.

Peptide Ser-TT$^{830-846}$ (**7**) (9 mg, 3.56 μmol) was dissolved in aqueous 0.1M KH_2PO_4, and the pH adjusted to 7 by adding 1N NaOH. $NaIO_4$ (3.3 mg, 1.2 equiv) dissolved in H_2O (300 μL) was then added, and the mixture was stirred at room temperature for 10 min and the reaction quenched by adding ethylene glycol (15 μL). The mixture was diluted with 50% aqueous AcOH, filtered, and purified by RP-HPLC [gradient: 100:0 to 80:20 (D/E), 7 min, then 80:20 to 75:25 (D/E), 18 min], to furnish **8** (5.5 mg, 72%) as a white powder after lyophilization; TOF-PDMS: *m/z*: 2059.1 $[M+Na]^+$.

H_2N-Gly-HA$^{307-319}$ (20): Following automated synthesis, one half of the peptidyl resin (0.125 mmol) was deprotected and coupled twice manually using a fourfold excess of preformed bromoacetic anhydride (prepared by DIC activation (20 min) in DMF), followed by washing with DMF (3×1 min) and CH_2Cl_2 (3×2 min). Then, *tert*-butylcarbazate (66 mg, 4 equiv) was added to the peptidyl resin, solvated in DMF containing DIEA (174 μL, 8 equiv). The mixture was stirred mechanically overnight and then washed with DMF (3×1 min), CH_2Cl_2 (3×2 min), and Et_2O (2×1 min). Finally, peptide was cleaved from the resin and deprotected with TFA/anisole/H_2O 95/2.5/2.5 (10 mL) for 3 h at room temperature, precipitated in cold diethyl ether, centrifuged, dissolved in water, and lyophilized. H_2N-Gly-HA$^{307-319}$ (**20**) (101 mg, 40%) was obtained as a white powder after semi-preparative RP-HPLC purification [gradient: 100:0 to 50:50 (A/F), 90 min], and lyophilization; positive ESI-MS: *m/z*: 1575 $[M+H]^+$.

H_2N-Gly-TT$^{830-846}$ (32a, 32b): After automated synthesis, peptidyl resin was deprotected and coupled twice manually using a fourfold excess of preformed bromoacetic anhydride (prepared by DIC activation (20 min) in DMF), followed by washing with DMF (3×1 min) and CH_2Cl_2 (3×2 min). Then, *tert*-butylcarbazate (66 mg, 4 equiv) was added to the peptidyl resin, solvated in DMF containing DIEA (174 μL, 8 equiv). The mixture was stirred mechanically overnight, and then washed with DMF (3×1 min), CH_2Cl_2 (3×2 min), and Et_2O (2×1 min). Finally, the peptide was cleaved from the resin and deprotected with TFA/iPr_3SiH/H_2O 95/2.5/2.5 (10 mL) for 1.5 h at room temperature, precipitated in cold diethyl ether, centrifuged, dissolved in water, and lyophilized. One half of the crude H_2N-Gly-TT$^{830-846}$ was purified by semi-preparative RP-HPLC [gradient: 100:0 to 75:25 (A/F), 25 min, then isocratic (A/F)] to furnish **32a** (51 mg, 15%) as a white powder after lyophilization; positive ESI-MS: found: 2052.0; calcd 2052.5; *m/z*: 1026.7 $[M+2H]^{2+}$, 684.8 $[M+3H]^{3+}$, 514.0 $[M+4H]^{4+}$. The other half of the crude peptide was purified by semi-preparative RP-HPLC [gradient: 100:0 to 80:20 (D/G), 50 min, then isocratic (D/G)] to furnish **35b** (30 mg, 11%) as a white powder after lyophilization.

H_2N-Gly-HSP65$^{3-14}$ (33): Peptide **33** was cleaved from the resin and deprotected with TFA/anisole/iPr_3SiH/H_2O 90/2.5/2.5/2.5 (10 mL) for 2 h at room temperature, precipitated in cold diethyl ether, centrifuged, dissolved

in H_2O/AcOH, and lyophilized. One quarter of the crude H_2N-Gly-HSP65[3–14] was purified by semipreparative RP-HPLC [gradient: 100:0 to 85:15 (A/F), 15 min, then 85:15 to 80:20 (A/F), 10 min, then isocratic (A/F)] to furnish **33** (39 mg, 32%) as a white powder after lyophilization; positive ESI-MS: found: 1479.0; calcd 1479.6; *m/z*: 740.5 $[M+2H]^{2+}$, 494.1 $[M+3H]^{3+}$.

General procedures for the double ligation: All solvents were degassed by bubbling N_2 through them before use.

Procedure A: Hydrazino- or α-oxo-aldehyde lysinyl tree (0.5–2 μmol) was treated overnight at room temperature under nitrogen with modified antigen peptide (1 equiv) in DMF/citrate-phosphate buffer pH 5 3:2 (500–1000 μL), with monitoring by RP-HPLC. In parallel, the disulfide derivative (1.5 equiv per chloroacetyl moiety to be substituted), dissolved in *n*PrOH/H_2O 1/1 (500 μL), was treated at room temperature overnight under nitrogen with tri-*n*-butylphosphane (1 equiv per disulfide). Completion of the reaction was monitored by RP-HLPC. The mixture was concentrated under reduced pressure for 20 min, and then dissolved in H_2O (100 μL) and added to the former reaction mixture, diluted with DMF (900–1400 μL, so as to obtain 80% aqueous DMF), and the apparent pH adjusted to 8–8.5 (paper) by addition of solid potassium carbonate. The mixture was stirred at room temperature for 24–48 h (monitoring by RP-HPLC), diluted with H_2O, frozen, and lyophilized. Compounds were obtained as white powders after RP-HPLC and lyophilization.

Procedure B: The disulfide derivative (1.5 equiv per chloracetyl moiety to be substituted), dissolved in *n*PrOH/H_2O 1/1 (500 μL), was treated overnight with tri-*n*-butylphosphane (1 equiv per disulfide) at room temperature under nitrogen. Completion of the reaction was monitored by RP-HLPC. The mixture was concentrated under reduced pressure for 20 min, and then dissolved in H_2O (50 μL) and added to 1–2 μmol of the glyoxylyl-lysinyl tree (1 equiv) dissolved in DMF (600 μL). The apparent pH was adjusted to 8–8.5 (paper) by addition of solid potassium carbonate. The mixture was stirred under nitrogen at room temperature for 24–48 h and monitored by RP-HPLC. Citrate/phosphate buffer (350 μL) and modified antigen peptide were then introduced. The pH was adjusted to 5.2 by addition of 1N aqueous HCl. The mixture was stirred for a further 12 h at room temperature, diluted with H_2O, frozen, and lyophilized. Compounds were obtained as white powders after RP-HPLC purification and lyophilization.

Procedure C: The disulfide derivative (1.5 equiv per chloroacetyl moiety to be substituted), dissolved in *n*PrOH/H_2O 1/1 (500 μL), was treated overnight with tri-*n*-butylphosphane (1 equiv per disulfide) at room temperature under nitrogen. Completion of the reaction was monitored by RP-HLPC. The mixture was concentrated under reduced pressure for 20 min. In parallel, 0.4 μmol of the glyoxylyl-lysinyl tree **16** was treated with the modified antigen (1 equiv) in *t*BuOH/H_2O (180–20 μL) at room temperature under nitrogen, and monitored by RP-HPLC. On completion, the reaction mixture was transferred to the crude thio derivative solution and the apparent pH adjusted to 8–8.5 (paper) by addition of solid potassium carbonate. After completion of the reaction, the crude mixture was diluted with H_2O, lyophilized and frozen, and lyophilized once more. Compounds were obtained as white powders after RP-HPLC and lyophilization.

Divalent conjugate 11: This compound (0.55 mg, 35%) was obtained following procedure A; RP-HPLC [gradient 100:0 to 80:20 (A/B), 10 min, then 80:20 to 70:30 (A/B), 40 min]; positive ESI-MS: *m/z*: 2976.2 $[M+K]^+$, 1469.8 $[M+2H]^{2+}$, 980.2 $[M+3H]^{3+}$, 735.4 $[M+4H]^{4+}$.

Tetravalent conjugate 12a: The double ligation was carried out on **5a**, which contained about 33% of untransformed lysinyl core **5b**, following procedure A. Compound **12a** (1.15 mg, 37%) was obtained, together with a more hydrophilic compound (**12b**, 0.91 mg, 52%) after RP-HPLC [gradient 100:0 to 80:20 (A/B), 10 min, then 80:20 to 70:30 (A/B), 40 min]; **12a**, positive ESI-MS: found: 3754; calcd 3755.43; *m/z*: 1878.7 $[M+2H]^{2+}$, 1252.6 $[M+3H]^{3+}$, 939.6 $[M+4H]^{4+}$; **12b**, positive ESI-MS: *m/z*: 1722.4 $[M+H]^+$, 861.5 $[M+2H]^{2+}$.

Divalent conjugate 21: This compound (1.18 mg, 43%) was obtained following procedure A; RP-HPLC [gradient 100:0 to 85:15 (A/B), 15 min, then 85:15 to 75:25 (A/B), 20 min, then isocratic]; positive ESI-MS: found: 2574; calcd 2575; *m/z*: 1288.2 $[M+2H]^{2+}$, 859.3 $[M+3H]^{3+}$, 644.7 $[M+4H]^{4+}$.

Tetravalent conjugate 22: This compound (1.49 mg, 40%) was obtained following procedure A; RP-HPLC [gradient 100:0 to 85:15 (A/B), 10 min, then 85:15 to 75:25 (A/B), 25 min, then isocratic]; positive ESI-MS: found: 3391; calcd 3392.0; *m/z*: 1696.5 $[M+2H]^{2+}$, 1131.5 $[M+3H]^{3+}$, 848.9 $[M+4H]^{4+}$.

Octavalent conjugate 24: This compound (1.99 mg, 18%) or (5.14 mg, 51%) was obtained following procedure A or B, respectively; RP-HPLC [gradient 100:0 to 85:15 (A/B), 15 min, then 85:15 to 75:25 (A/B), 20 min, then isocratic]; positive ESI-MS: found: 5025; calcd 5025.9; *m/z*: 1676.0 $[M+3H]^{3+}$, 1257.3 $[M+4H]^{4+}$.

Octavalent conjugate 30: This compound (0.90 mg, 41%) was obtained following procedure B; RP-HPLC [gradient 100:0 to 85:15 (A/B), 15 min, then 85:15 to 72:28 (A/B), 25 min, then isocratic]; positive ESI-MS: found: 5024.0; calcd 5025.9; *m/z*: 1257.5 $[M+4H]^{4+}$, 1006.1 $[M+5H]^{5+}$.

Octavalent conjugate 31: This compound (1.52 mg, 35%) was obtained following procedure B; RP-HPLC [gradient 100:0 to 90:10 (A/B), 10 min, then 90:10 to 75:25 (A/B), 50 min]; positive ESI-MS: found: 5112; calcd 5114.1; *m/z*: 1704.9 $[M+3H]^{3+}$, 1278.9 $[M+4H]^{4+}$, 1030.9 $[M+5H+K]^{5+}$, 859.3 $[M+5H+K]^{6+}$.

Octavalent conjugate 34: This compound (0.40 mg, 45%) was obtained following procedure B; RP-HPLC [gradient 100:0 to 75:25 (A/B), 15 min, then 75:25 to 70:30 (A/B), 30 min]; positive ESI-MS: found: 4927.5; calcd 4928.6; *m/z*: 1643.7 $[M+3H]^{3+}$, 1233.2 $[M+4H]^{4+}$, 999.3 $[M+3H+Na+K]^{5+}$.

Tetravalent conjugate 35: This compound (0.45 mg, 59%) was obtained following procedure C; RP-HPLC [gradient 100:0 to 60:40 (A/B), 70 min]; positive ESI-MS: found: 3867; calcd 3869.5; *m/z*: 1290.1 $[M+3H]^{3+}$, 968.0 $[M+4H]^{4+}$, 774.6 $[M+5H]^{5+}$.

Tetravalent conjugate 36: This compound (0.55 mg, 35%) was obtained following procedure C; RP-HPLC [gradient 100:0 to 75:25 (A/B), 20 min, then 75:25 to 65:35 (A/B), 20 min, then isocratic]; positive ESI-MS: found: 3868.5; calcd 3869.5; *m/z*: 1290.6 $[M+3H]^{3+}$, 968.0 $[M+4H]^{4+}$, 783.6 $[M+5H]^{5+}$.

Tetravalent conjugate 37: This compound (0.71 mg, 42%) was obtained following procedure C; RP-HPLC [gradient 100:0 to 75:25 (A/B), 20 min, then 75:25 to 60:40 (A/B), 30 min]; positive ESI-MS: found: 3912; calcd 3913.6; *m/z*: 1305.0 $[M+3H]^{3+}$, 979.1 $[M+4H]^{4+}$, 783.6 $[M+5H]^{5+}$.

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