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Orthogonal solid-phase synthesis of tetramannosylated peptide constructs carrying three independent branched epitopes

Goran Kragol and Laszlo Otvos, Jr.*

The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104, USA

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Abstract—The development of peptide-based drugs requires the chemical synthesis of systems as complex as they appear in nature. Most bioactive peptides have to be associated with co-activators and delivery or targeting domains, the synthesis of such complexes is far from trivial. In efforts to develop a prototype for a new generation of peptide vaccines, a peptide construct was prepared, using an alternating lysine and glycine backbone. A 24-mer major antigen corresponding to the M2 protein of influenza virus, and two shorter T-cell epitopes derived from the hemagglutinin were co-synthesized onto the side chains of the first three lysines. To help the delivery of the constructs inside the antigen presenting cells via the multimeric cell surface mannose receptor, three additional lysines were decorated with four mannosylated serine residues. The synthesis difficulty increased upon addition of the glycoamino acids and alternating the peptide and glycoamino acid branches. The successful solid-phase synthesis of the constructs proceeded with the use of a combination of three quasi-orthogonally removable amino protecting groups and a robust activation strategy. These multi-glycosylated constructs represent some of the most complex synthetic peptides to date, and will be used to study the entire process of antigen delivery, presentation and immunogenicity.

1. Introduction

Peptide fragments represent the biologically active domains of native proteins. In spite of demonstrating activities comparable with full-sized proteins, synthetic peptides are rarely considered as viable drug candidates. This is partly because of the proteolytic instability[1] or sometimes disadvantageous pharmacokinetic properties of peptides.[2] Frequently a short protein fragment, eliciting a well-characterized in vitro biological response, lacks additional properties that will enable successful in vivo and therapeutic utilization. First of all many recognition processes require the presence of multiple independent protein fragments, co-activators or chaperones.[3] Second, it is generally considered that peptides without carrier modules are unable to enter cells,[4] although this latter assumption is now increasingly challenged.[5] Based upon these considerations, synthetic peptide constructs designed for therapeutic use need to contain determinants for the target biological activity, bystander activators and delivery modules.

This triad requirement is most obvious for the development of subunit peptide vaccines. Successful immunizations with peptide antigens can only be achieved if at least one T-helper cell determinant is included in the immunogen construct,[6] or in the immunizing cocktail.[7] Multiple peptide epitopes, representing determinants of more than one major histocompatibility complex (MHC) proteins, are used to overcome unresponsiveness observed in outbred animals and in humans, or improve antigen presentation in inbred animals.[8] While earlier strategies preferred a co-linear assembly of the chimeric T-B determinant peptide construct[9] most recent data support a branched-type arrangement of the peptide constituents.[10,11] A major obstacle to subunit vaccines is that synthetic peptides are presented inefficiently for interaction with MHC proteins. External peptide antigens bind to the limited number of 'empty' MHC proteins that reach the cell surface, in contrast with natural antigen processing that presents peptide fragments for MHC interaction inside the cells. It is well known that mannosylation of proteins can facilitate their entry into cells via the mannose binding protein at the surface of cells,[12] and indeed, mannose receptor-mediated uptake enhances MHC class II-restricted peptide antigen presentation by cultured dendritic cells.[13] A nice example of carbohydrate–peptide combination used in vaccine development can be found in fully synthetic glycopeptide-based anticancer vaccines.[14]

All these considerations led us to the design of special constructs during our quest to develop peptide vaccines to influenza viruses (Scheme 1). In contrast to influenza virus glycoprotein vaccines, the 24-mer ektodomain (SLLTEVETPIRNEWGCRSNDSSDP) of the transmembrane ion-channel protein M2 has the potential advantage of providing highly crossreactive protection.[15–18] For T-helper cell epitopes we selected I-E^d restricted S1 peptide[19,20]

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* Corresponding author. Tel.: +1-215-898-3772; fax: +1-215-898-5821; e-mail: otvos@wistar.upenn.edu

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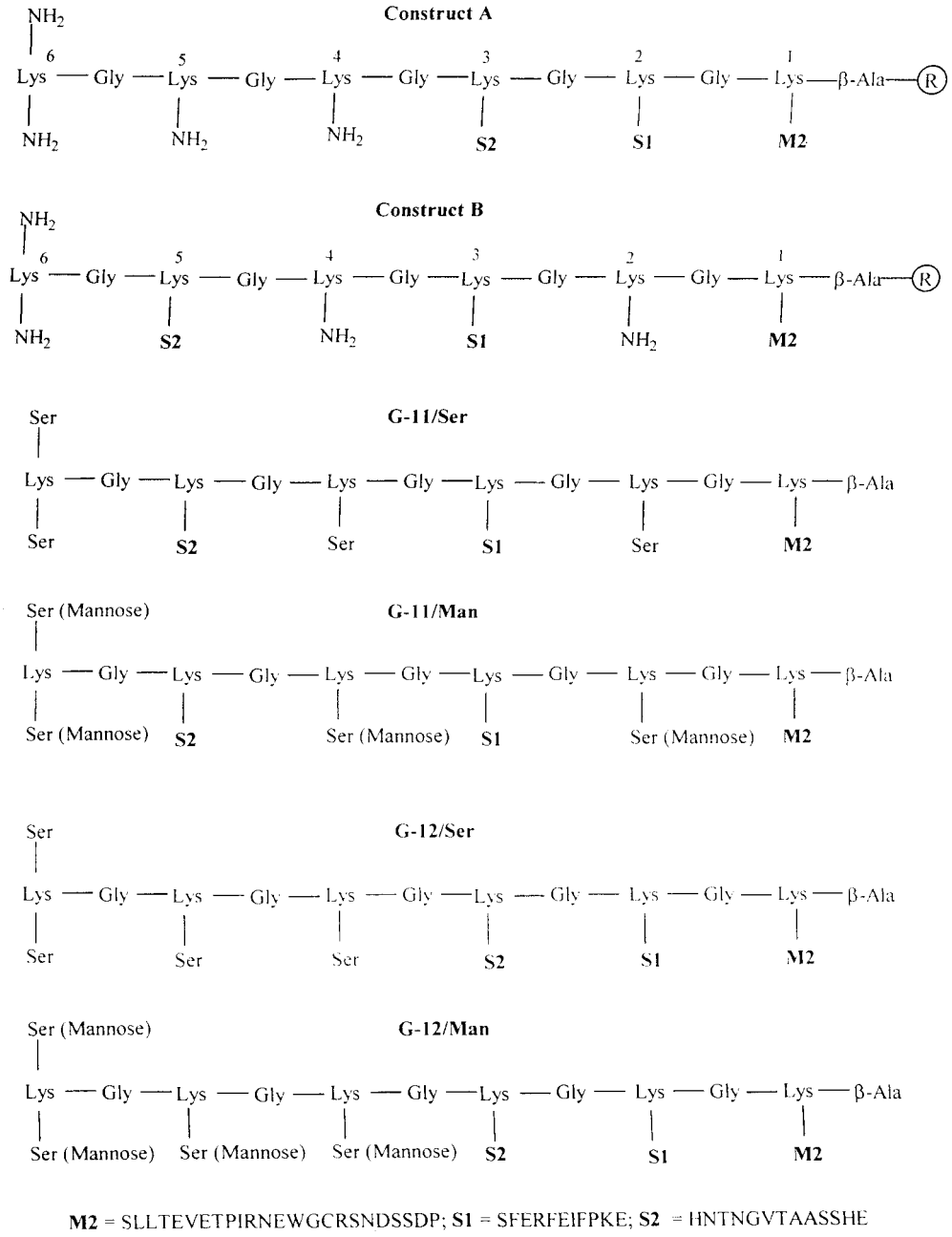
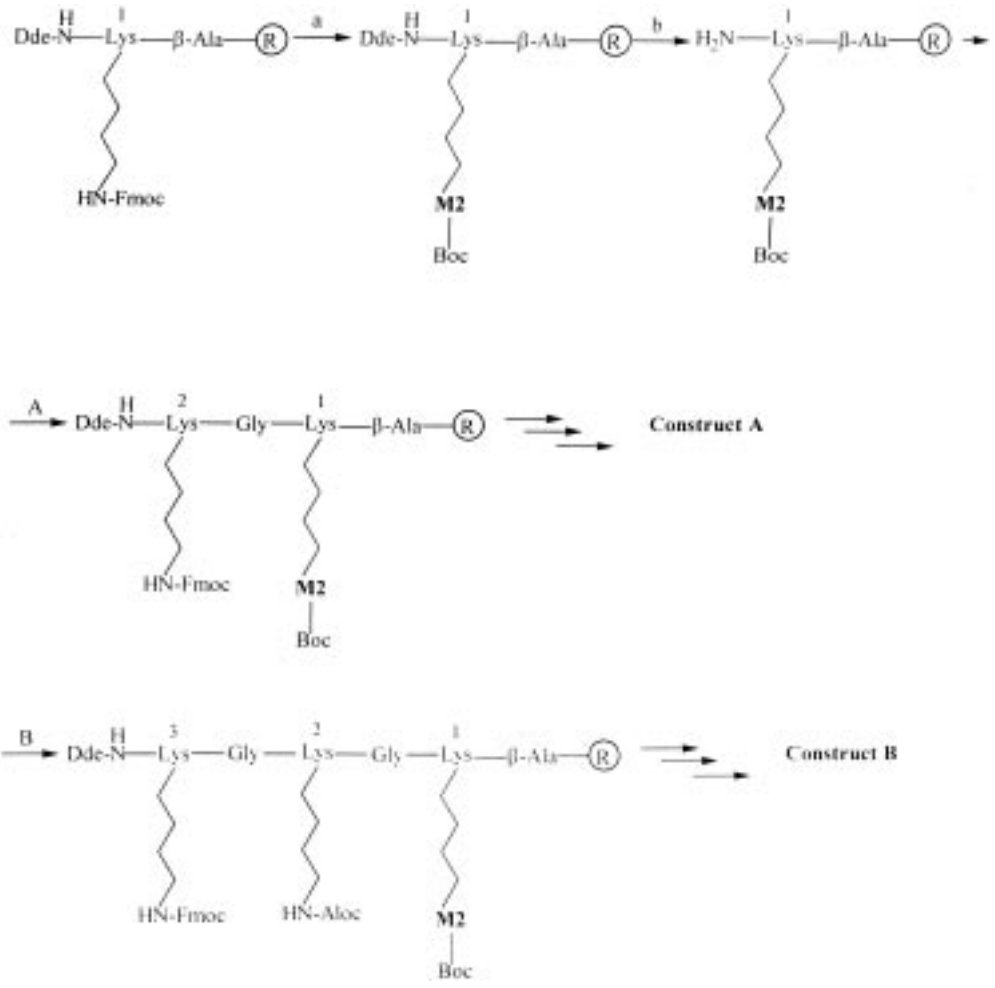


Scheme 1.

SFERFEIFPKE and I-A^{d} restricted S2 peptide[20,21] HNTNGVTAASSHE which are the two dominant helper determinants of the hemaglutinin molecule of influenza virus A/PR/8/34. Finally, for targeting the mannose receptor, which preferentially binds branched chain mannose oligosaccharides,[22] we chose four copies of D-mannose linked to carrier serine residues through the anomeric carbon atom. The four mannoses were positioned either close to each other or interspersed with the T-helper cell epitopes to gain information of the relative spatial requirements of the sugar and the peptides. The corresponding non-glycosylated peptides were also prepared. We retained the serine residue in the strategical positions because of their advantageous physical and biological properties on subunit vaccine carriers.[23] Here we report the preparation of the glycoamino acid and the various peptide and glycopeptide constructs. From the biological perspective these constructs represent the prototypes of a new generation of peptide vaccines. From a chemical standpoint these reactions exemplify the state-of-the-art of solid-phase glycopeptide synthesis.

2. Results and discussion

The preparation of multivalent mannosylated and non-mannosylated constructs G-12 and G-11 started with the synthesis of resin-bound polylysine–polyglycine mixed backbones A and B that carried the peptide epitopes (Scheme 2). The B-cell epitope M2 and the T-helper cell



Scheme 2. (a) Fmoc solid phase strategy, Boc-Ser(tBu)-OH coupled as the terminal residue of M2; (b) 2% hydrazine solution in DMF.

epitopes S1 and S2 were synthesized onto the side chains of three ε-amino groups of the polylysine–glycine backbone as the backbone grew rather than after the complete assembly of the polylysine–polyglycine stretch. The amino termini of the epitopic peptide domains were protected with *t*-butyloxycarbonyl (Boc). The two additional internal lysines and the third one at the amino termini of the backbone structures remained without amino terminal protection, and served as carriers for naked and mannosylated serine residues. Subsequent coupling of the four Fmoc-Ser(^{t}Bu)-OH or Fmoc-Ser(Ac_4-Man)-OH gave the final constructs G-11 and G-12. The polylysine–polyglycine mixed backbones A and B were prepared by continuous flow solid-phase peptide synthesis on an Fmoc–PAL–polyethyleneglycol–polystyrene resin with relatively low initial capacity (c=0.15 mmol g^{-1}) to increase amino acid coupling rates due to resin-induced pseudo-dilution effects.[24] Actually this design was borne out of the multiple antigen peptide concept[25] with the advantage of increased control over the spacing of the antigenic determinants. It needs to be added that multiple epitopes can also be built on the conventional multiple antigenic peptide core,[26] and that a two-dimensional multiple antigenic peptide construct, utilizing our principal selections, the 9-fluorenylmethoxycarbonyl (Fmoc) and 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) protecting groups, was used to prepare lipid-conjugated peptide immunogens.[27]

2.1. Preparation of the activated glycoamino acid

For mannose incorporation into constructs A and B we decided to use *N*-Fmoc-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-L-serine [Fmoc-Ser(Ac_4-Man)-OH] and/or its pentafluorophenyl ester [Fmoc-Ser(Ac_4-Man)-OPfp]. The sugar hydroxyl groups were protected with acetylation, not only to prevent unwanted acylation during the peptide assembly, but also because unprotected carbohydrates, although they are less sterically demanding, are increasingly susceptible to cleavage of the glycosidic bond during prolonged (>2 h) acidic removal of the final glycopeptide from the resin, especially in the presence of water.[28] Fmoc-Ser(Ac_4-Man)-OH was prepared by the reaction of α-D-mannose pentaacetate with Fmoc-Ser-OH in the presence of 9 equiv. of boron trifluoride etherate in dichloromethane for 20 h, similarly to the synthesis of the corresponding mannosylated threonine residue.[29] After working out the optimal reaction conditions on a small scale (~50 mg) and the product analysis by thin layer chromatography (silica gel, methanol/dichloromethane, 1:9 v/v), the reaction was scaled up to 2.5 g. The protected glycoamino acid was

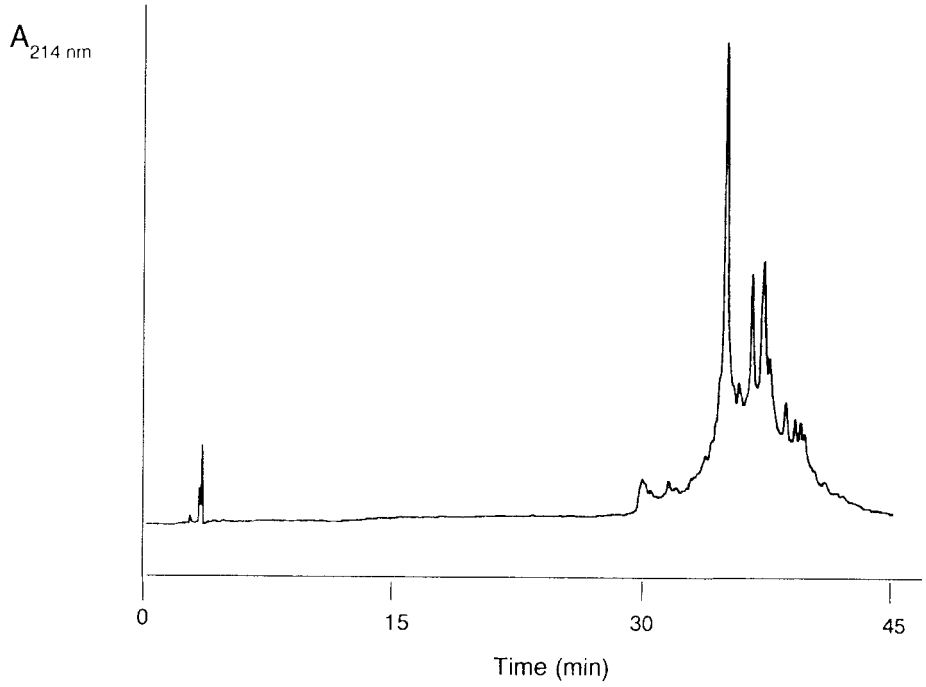


Figure 1. Reversed-phase high performance liquid chromatogram of the polylysine–polyglycine backbone structure construct A that carried peptides M2, S1, and S2 at the side chains of lysines 1, 2, and 3. The chromatogram was developed with gradient 1.

purified by flash chromatography on silica gel providing the final product with good yield (70%). ^{13}C and ^{1}H NMR spectra were in accordance with published data for Fmoc-Ser(Ac_4-Man)-OH.[30] The activated derivative was prepared by reaction of Fmoc-Ser(Ac_4-Man)-OH with 1.1 equiv. of pentafluorophenol and diisopropylcarbodiimide in 93% yield. This highly economical synthetic strategy furnished the target mannosylated serine building blocks, ready for the solid-phase synthesis of glycopeptides, in a large scale needed for the preparation of constructs G-11/Man and G-12/Man.

2.2. Synthesis of construct A

Construct A was prepared in a few consecutive steps using two different protective groups for the lysines. The Dde group was used for protection of the α-amino function, while the ε-amino groups of lysines 1, 2, and 3 were protected with Fmoc. Lysines 4 and 5 were introduced as Fmoc–Lys(ivDde)–OH and the amino terminal lysine 6 was coupled as Fmoc–Lys(Fmoc)–OH. The first step in the synthesis was coupling of β-Ala to the Fmoc–PAL–PEG–PS resin and subsequent addition of lysine 1 as Dde–Lys(Fmoc)–OH. After removal of the side chain protecting Fmoc group, the M2 epitopic peptide was synthesized onto lysine 1 with its amino-terminal Ser added as Boc-Ser(^{t}Bu)-OH. After the assembly of M2 was completed, the amino-terminal Dde protecting group of lysine 1 was removed manually using 2% hydrazine dissolved in dimethylformamide (DMF). The construction proceeded on the synthesizer with an identical strategy until both T-helper cell epitopes S1 and S2 were incorporated. After removal of the amino-terminal Dde protection from lysine 3, the rest of the backbone, Lys6–Gly–Lys5–Gly–Lys4–Gly, was made by using standard Fmoc protocols. The two Fmoc groups from lysine 6 were cleaved with 20% piperidine in DMF using the synthesizer's end cycle deprotection program, and then the two ε-amino protecting Dde groups from lysines 4 and 5 were cleaved manually with 2% hydrazine solution in DMF. This synthetic strategy yielded the final construct A (Path A on Scheme 2). We checked the purity of the construct before the incorporation of the glycosylated mannose derivative. A small sample was removed from the solid carrier with trifluoroacetic acid (TFA):water:thioanisole (90:5:5, v/v/v), and was analyzed with RP-HPLC. The chromatogram showed one main peak and a few additional ones, in much smaller quantities (Fig. 1).

2.3. Preparation of mannosylated and non-mannosylated constructs G-12

The addition of the four serines to construct A was achieved by coupling 4 equiv. (calculated to the number of free amino groups of the resin-bound lysine residues) of Fmoc-Ser(^{t}Bu)-OH to lysines 4, 5, and 6 with prolonged (4 h) coupling time on the synthesizer. The end cycle program was used to remove the four Fmoc protecting groups from the serine amino functions. Cleavage of the peptide from resin was completed with a 4 h treatment with a TFA: thioanisole:water (90:5:5, v/v/v) mixture. All these final reactions proceeded smoothly, and analytical high performance liquid chromatography (HPLC) of the crude product showed one main peak. The final construct G-12/Ser could be easily isolated and purified using preparative reversed-phase (RP)-HPLC. Matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) of the purified peptide G-12/Ser showed the expected mass at *m/z* 6895.

Mannosylation of construct A was first attempted by

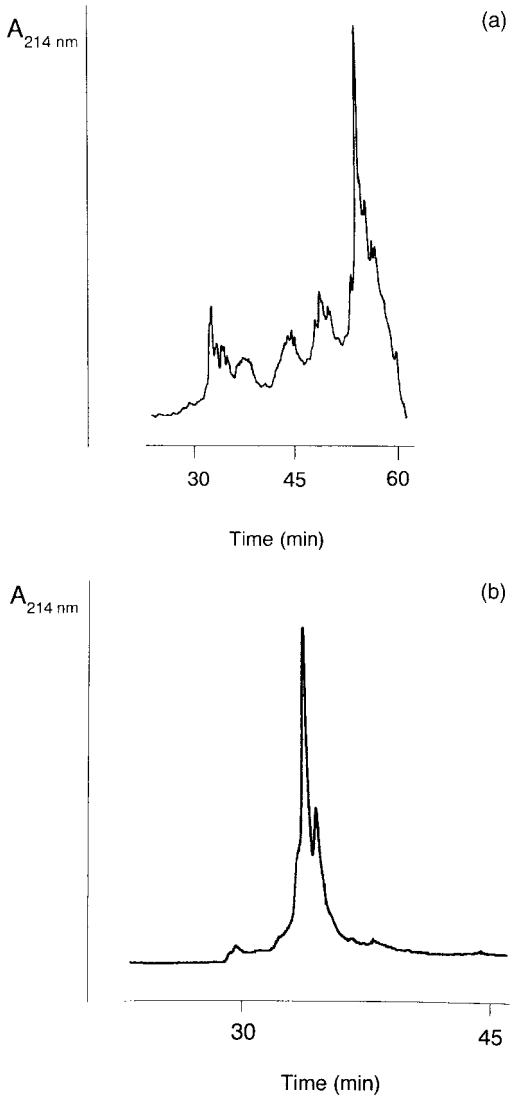


Figure 2. Reversed-phase high performance liquid chromatograms during the preparation of G-12/Man: (a) crude product after incorporation of four Fmoc-Ser(Ac_4-Man)-OH; (b) deprotected sample from which the final purification of G-12/Man proceeded.

coupling 2 equiv. (calculated to the available free amino groups) of Fmoc-Ser(Ac_4-Man)-OPfp in DMF. The progress of the reaction was monitored with the ninhydrin test.[31] When the coupling step appeared to be completed, the peptide was cleaved from the resin with a 4 h treatment with the usual TFA:thioanisole:water (90:5:5, v/v/v) cleavage mixture. A series of chromatographic runs and mass spectra indicated that although the crude product contained the target glycopeptide, its quality was insufficient for obtaining a clean final material. Therefore, we changed the synthetic strategy, and incorporated Fmoc-Ser(Ac_4-Man)-OH with *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate (HATU) activation. HATU is recommended for the synthesis of complex peptides.[32] The coupling reaction was stopped after 3 days. To find out the best method for the cleavage of the glycopeptide from the resin, the TFA:water:thioanisole, 90:5:5 (v/v/v) cocktail was compared with reagent K (TFA:water:phenol:thioanisole:ethanedithiol, 82.5:5:5:5:2.5, v/v/v/v/v).[33] In both cases, the cleavage time was 4 h. According to analytical RP-HPLC, the two cleavage mixtures provided almost identical crude products (Fig. 2(a)). MALDI-MS of the purified glycopeptide, which was still protected with four Fmoc groups and 16 acetyl groups, showed the expected molecular peak at *m/z* 9108. From our preliminary studies with acetylated complex glycopeptides we know that the difficulty of removing the acetyl groups increases with increasing sterical hindrance. To find out the minimal time required for complete acetyl removal, small aliquots were taken, treated with 0.1M NaOH for 10, 30, and 45 min, and completion of the hydrolysis was monitored with RP-HPLC and MALDI-MS. According to these studies, a period of at least 30 min was needed to remove all acetyl groups (Fig. 2(b)). Although strong basic conditions can result in β-elimination, required deprotection time is still five times shorter than that known to cause β-elimination during sodium hydroxide deprotection.[34] The final glycopeptide was purified on a semi-preparative RP-HPLC column. Analytical HPLC showed a single peak (Fig. 3) and MALDI-MS revealed a remarkably pure product considering the size and complexity of this mannosylated construct (Fig. 3 inset). In accordance with the general rules,[35] the glycopeptide exhibited a shorter retention time than its non-glycosylated parent analog (Table 1).

2.4. Preparation of construct B

The level of difficulty further increased for construct B because the ε-amino groups of lysines 2 and 4 had to remain protected until the end of the construction. Consequently, we used an orthogonal amino function protecting strategy, with the Pd^0 removable allyloxycarbonyl (Aloc) group to the third dimension, and applied this for lysines 2 and 4. Lysines 1, 3, and 5 were coupled as Dde–Lys(Fmoc)–OH while the amino-terminal lysine 6 was coupled as Fmoc–Lys(Fmoc)–OH. Other than this alteration, the general strategy for the synthesis of construct B was identical to that for the synthesis of construct A (Path B on Scheme 2). Because removal of the Dde group with 2% hydrazine solution in DMF can result in partially hydrogenated Aloc groups, removal of the Dde protection was done with 2% hydrazine and 1% allyl alcohol (as a scavenger) solution in DMF.[36] After addition of the amino-terminal lysine 6, the Fmoc groups from this last lysine were removed with 20% piperidine in DMF using the end cycle deprotection program of the automated synthesizer. The lysine 2 and 4 side chain protecting Aloc groups were removed manually by shaking the resin-bound peptide with 3 equiv. of $(PPh_3)_4Pd$ in chloroform:acetic acid:*N*-methyl morpholine, 37:2:1, v/v/v in a glass vial for 5 h. After cleavage from the resin using TFA:water:thioanisole, 90:5:5 (v/v/v) of a small aliquot of construct B, analytical RP-HPLC showed one main peak. However, a considerably increased level of closely eluting contaminating peaks was observed in comparison with construct A, reflecting the increased level of difficulty of the synthesis.

2.5. Preparation of mannosylated and non-mannosylated constructs G-11

Fmoc-Ser(^{t}Bu)-OH was incorporated into construct B in conditions identical to those for construct A. After cleavage

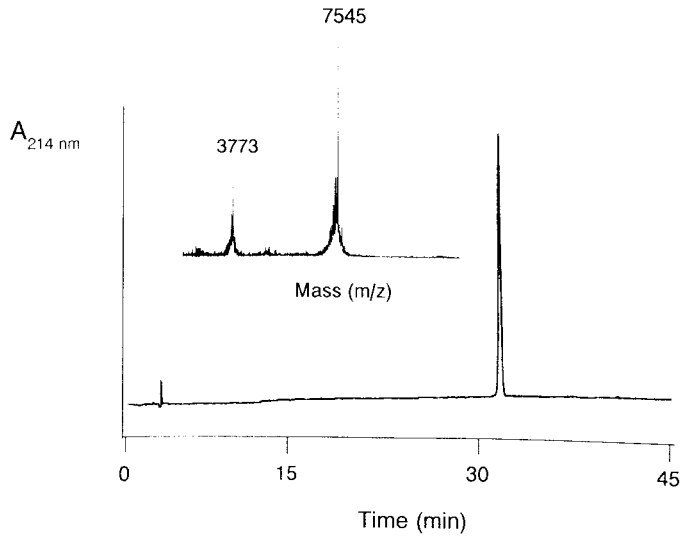


Figure 3. Reversed-phase high performance liquid chromatogram of the purified tetramannosylated construct G-12/Man. Gradient 1 was used. The inset shows the matrix-assisted laser ionization/desorption mass spectrum of the same sample. The peak at *m/z* 7545 corresponds to the expected molecular ion, the peak at *m/z* 3773 represents the double charged ion.

Table 1. Analytical data of the synthetic peptide constructs

Construct	RP-HPLC retention time (min) using gradient 1	MALDI-MS [(M+H)$^+$]	
		Calculated	Observed
G-12/Ser	34.7	6893	6895
G-11/Ser	34.4	6893	6894
G-12/Man protected	56.7	9108	9106
G-11/Man protected	54.6	9108	–
G-12/Man	33.9	7545	7545
G-11/Man	34.7	7545	7542

of the peptide from the resin, one main peak was detected on analytical RP-HPLC, with the addition of a few more hydrophobic contaminants. This crude mixture could be easily purified on RP-HPLC to yield a seemingly clean peptide (Fig. 4). However, on the mass spectrum of the purified product G-11/Ser, the major, expected molecular ion at *m/z* 6894 was accompanied by contaminants in smaller quantities (Fig. 4 inset). A thorough analysis of the mass spectra revealed that the major contaminating peaks corresponded to peptides with one and two serine residues missing from the sequence. Apparently RP-HPLC was unable to resolve the crude peptide mixture. This is understandable in light of the minimal contribution serine makes to the

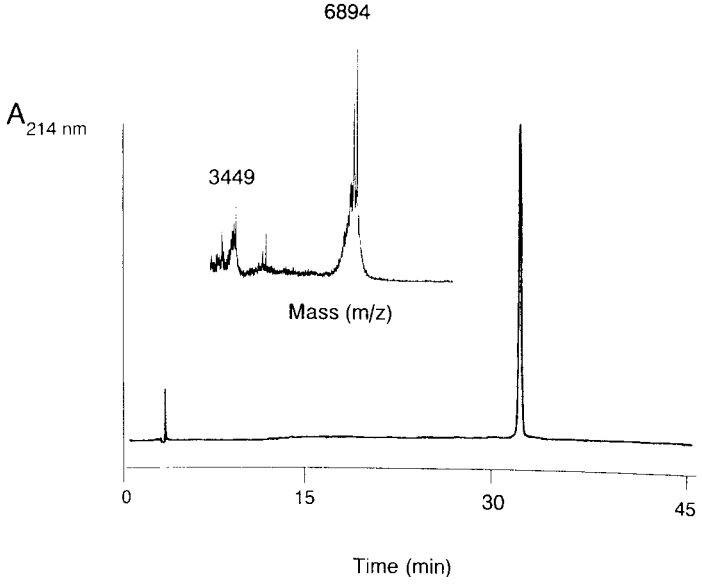


Figure 4. Reversed-phase high performance liquid chromatogram of the purified non-mannosylated construct G-11/Ser. The inset shows the matrix-assisted laser ionization/desorption mass spectrum of the same sample. The peak at *m/z* 6894 corresponds to the expected molecular ion, the peak at *m/z* 3449 represents the double charged ion. Main side products are peptides with one and two serine residues missing.

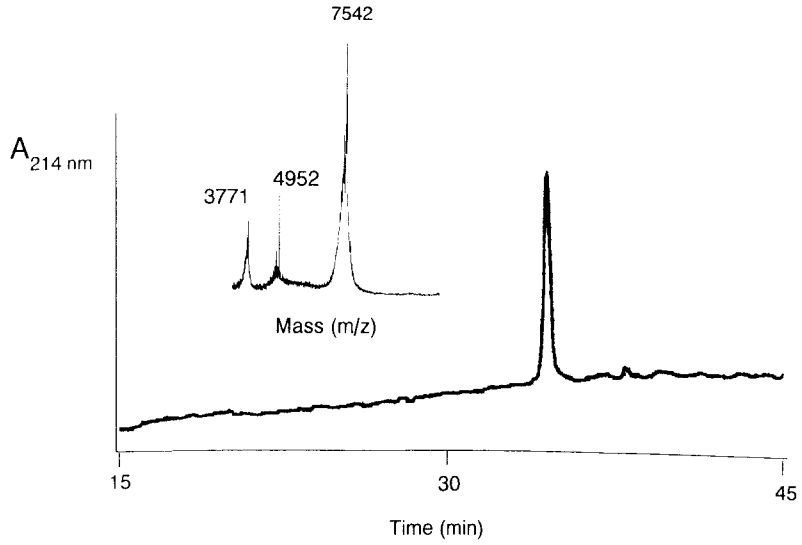


Figure 5. Reversed-phase high performance liquid chromatogram of the purified tetramannosylated construct G-11/Man. The inset shows the matrix-assisted laser ionization/desorption mass spectrum of the same sample. The peak at *m/z* 7542 corresponds to the expected molecular ion, the peak at *m/z* 3771 represents the double charged ion. Probable main side products are peptides with one and two mannosylated serine residues missing. The peak at *m/z* 4952 represents a side product of the synthesis of backbone construct B.

reversed-phase retention time of peptides in the TFA/acetonitrile elution system,[37] the terminal location of the serines and the size of the peptide construct. The inefficient coupling of the serine residues into the side chains of backbone lysines 2 and 4, located between the peptide chains M2, S1, and S2 indicated the significant steric hindrance around the target lysine residues. This situation is similar to our unsuccessful attempts to glycosylate aspartic acid residues with maltoheptosylamine next to residues that were backbone protected.[38]

The preparation of the construct G-11/Man was attempted by coupling of Fmoc-Ser(Ac_4-Man)-OPfp to construct B, as well as addition of Fmoc-Ser(Ac_4-Man)-OH with HATU activation. The reaction conditions remained identical to those described for construct G-12/Man except 1 equiv. of HOBt was used as an additive for the coupling Fmoc-Ser(Ac_4-Man)-OPfp as it is known that HOBt can accelerate the coupling rates of pentafluorophenyl esters.[39] However, neither of these coupling reactions resulted in any peak for which the RP-HPLC retention time would have suggested the presence of the intended glycopeptide. The lack of the target compound was further verified by the lack of the expected molecular ion from the mass spectrum of the cleaved product. If the incorporation of the serine with *tert*-butyl side chain protection was difficult between the epitopic peptide chains for construct G-11/Ser, the addition of the significantly bulkier mannosylated serine derivative into construct G-11/Man was expected to be even more troublesome. This was further supported by the decreased coupling yields of the mannosylated serine compared to the naked one into the somewhat simpler G-12 constructs. Based on these observations, a third try of the mannosylation of construct B was done by using four molar excess of Fmoc-Ser(Ac_4-Man)-OH activated in situ with HATU and employing double coupling cycles (2×6 h) on a batch mixing synthesizer. Analytical RP-HPLC (gradient 1) of the crude peptide mixture showed a small peak with a retention time in the proximity of that of Fmoc and acetyl protected G-12/Man. Because the isolation of the highly hydrophobic protected glycopeptide and characterization of the reaction mixture by mass spectrometry appeared to be overly inconvenient, all the base labile protecting groups were removed with a 30 min treatment with 0.1M NaOH, and the glycopeptide was purified in the deprotected form. For this purification an extremely shallow gradient was applied (0.2% acetonitrile per minute), the one that yielded a remarkably pure sample of the synthetic 82-mer antibacterial peptide diptericin.[40] This maneuver resulted in a single, albeit somewhat broad, peak on the reversed-phase chromatogram (Fig. 5). In spite of the identical overall chemical composition, the retention time of the G-11/Man construct was 0.8 min delayed compared to that of G-12/Man, and actually was longer than that of the non-glycosylated parent analog G-11/Ser (Table 1). This clearly demonstrated the shielding of the hydrophilic mid-chain carbohydrate moieties. The MALDI mass spectrum featured the expected molecular ion at *m/z* 7542, the double charged form at *m/z* 3771, some deleted products of almost similar size and a shorter contaminant at *m/z* 4952 (Fig. 5 inset). This last side product was derived from the synthesis of the base construct B, as the same molecular ion side product=*m/z* 2590 peak was also detectable in the mass spectrum of non-mannosylated construct G-11/Ser (cf. the inset of Fig. 4). Although the purity grade of construct G-11/Man seemed to be above the acceptable level of a peptide construct of this complexity, it remained below the purity degree of the non-alternating sister construct G-12/Man.

3. Conclusion

Taken together we successfully synthesized the target tetramannosylated peptide constructs that carried three independent epitopic peptide branches. These glycopeptides represent some of the most complex synthetic peptides

made to date. Although the presented reaction scheme will be unable to provide drug leads in quantities large enough for widespread application, the final products represent the prototypes of a new generation of therapeutic peptides. In particular, these glycopeptide constructs will be suitable in quantity and quality to investigate basic antigen presentation and delivery phenomena and to assess the potential of these compounds as a novel type of synthetic subunit vaccines. Preliminary immunological results indicate that both the topology of the epitopes and the presence of the mannose moieties influence the immunogenicity of the peptide constructs. Virus-specific T-cell production in immunized BALB/c mice showed that constructs G-12/Ser, G-12/Man, and G-11/Man were as immunogenic as the influenza virus A/PR/8/34 itself. In contrast, construct G11/Ser without added mannose moieties elicited very poor immune response. A detailed analysis of the T-cell as well as antibody repertoire upon immunization with these peptides will be available when the experiments are repeated on a statistically satisfactory number of mice and tested against a long range of antigens. Ultimately, the immunized mice will be tested for the ability to fight off viral challenge.

4. Experimental

4.1. Analytical techniques

All synthetic compounds were analyzed and purified by RP-HPLC using a Waters low-pressure mixing gradient system and Phenomenex analytical and preparative Jupiter C18 columns. Solvent A contained 0.1% TFA in water and solvent B contained 0.1% TFA in acetonitrile. All analytical and preparative runs consisted of a 5 min isocratic elution of 5% B (gradient 1) or 20% B (gradient 2) followed by a linear gradient from 5 to 65% B or 20 to 80% B for 45 min and then again isocratic elution of 65 or 80% B, respectively. The amide bond was detected at 214 nm. MALDI-MS was done at the Wistar Institute Protein Microchemistry Laboratory on a Voyager Biospectrometry Workstation by standard methods. NMR spectra were acquired on a Bruker 500 MHz spectrometer.

4.1.1. Synthesis of *N*-Fmoc-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-L-serine. α-D-Mannose pentaacetate (1.06 g, 2.72 mmol) and Fmoc-Ser-OH (1.08 g, 3.28 mmol) were dissolved in 50 ml dichloromethane and 3.1 ml (24.6 mmol) boron trifluoride etherate was added. The reaction mixture was stirred for 20 h in dry atmosphere (fitted with a $CaCl_2$ tube), diluted with 160 ml dichloromethane and washed with 20 ml of 1M HCl and 20 ml water. The organic phase was dried over anhydrous $MgSO_4$. After evaporation of the solvent on a rotary evaporator and purification of the crude mixture (2.03 g) by flash chromatography on silica gel (eluent:methanol: dichloromethane, 5:95, v/v), 1.25 g (70%) of product, the protected glycoamino acid, was obtained (~90% pure based on analytical RP-HPLC, gradient 2). ^1H and ^{13}C NMR spectra (in $CDCl_3$) were in accordance with published data for *N*-Fmoc-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-L-serine.

4.1.2. Synthesis of *N*-Fmoc-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-L-serine pentafluorophenyl ester. The solution of 0.25 g (0.38 mmol) *N*-Fmoc-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-L-serine, 0.077 g (0.42 mmol) pentafluorophenol and 0.066 ml (0.42 mmol) diisopropylcarbodiimide in 50 ml dichloromethane was stirred for 3 h at room temperature in dry atmosphere (fitted with a $CaCl_2$ tube). The reaction mixture was washed with 5 ml water, dried over anhydrous $MgSO_4$ and concentrated on a rotary evaporator. One hundred ml of petroleum ether was added and the precipitated *N*-Fmoc-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-L-serine pentafluorophenyl ester was filtered. The yield was 0.29 g (93%). According to analytical RP-HPLC, gradient 2, the product was ~95% pure. MALDI-MS of the purified sample showed peaks at *m/z* 845 corresponding to $(M+Na)^+$ and at *m/z* 861 corresponding to $(M+K)^+$.

4.1.3. Assembly of constructs A and B. Assembly of constructs A and B was accomplished on a continuous flow automated peptide synthesizer (Milligen 9050) utilizing conventional Fmoc chemistry.[41] For chain elongation, a four molar excess of the amino acids was activated in situ with HATU. From the amino-protecting groups, Fmoc was removed on the synthesizer, and Dde and Aloc were removed manually. Constructs A and B were synthesized on 1 g Fmoc–PAL–PEG–PS resin (Perseptive Biosystems, Warrington, UK) with an initial load of 0.15 mmol g^{-1}, yielding C-terminal amides after TFA cleavage. The coupling times ranged from 1 to 2.5 h according to coupling difficulties predicted by the Peptide Companion (Windowchem, Fairfield, CA) algorithm.

4.1.4. Dde group cleavage. The peptide resin was transferred from the synthesizer to a small flask fitted with a stopper. The solution of 2% hydrazine in DMF (during the synthesis of construct A) or 2% hydrazine and 1% allyl alcohol (in the case of construct B), was added. The flask was stoppered and after 3 min the hydrazine solution was decanted. This treatment was repeated twice, and the resin was filtered and washed with DMF.

4.1.5. Aloc group cleavage. An amount of 0.35 g peptide resin was placed in a glass vial and a solution of 1 g $(PPh_3)_4Pd$ in 10 ml chloroform:acetic acid:*N*-methyl morpholine (37:2:1, v/v/v) mixture was added. The reaction mixture was shaken for 5 h, the resin was filtered and washed twice with 25 ml of 0.5% diisopropylethylamine dissolved in DMF, twice with 25 ml of 0.5% sodium diethyldithiocarbamate also dissolved in DMF and finally twice with 25 ml DMF alone.

4.1.6. Synthesis of constructs G-12/Ser and G-11/Ser. A four molar excess of Fmoc-Ser(^{t}Bu)-OH (0.14 g, 0.36 mmol for G-12/Ser and 0.10 g, 0.26 mmol for G-11/Ser, calculated to the available free amino groups) was activated with HATU and was introduced with extended coupling times (4 h) into constructs A (made on 0.15 g of resin, 0.15 mmol g^{-1}) and B (made on 0.11 g of resin, 0.15 mmol g^{-1}) on the continuous flow Milligen 9050 automated synthesizer. Peptides were cleaved off the solid support by shaking the peptide resins with 90:5:5 (v/v/v) mixture of TFA:water:thioanisole (2.5 ml per 0.1 g of

resin) in a glass vial for 4 h. After precipitation into cold ether, the peptides were centrifuged, filtered and purified using preparative RP-HPLC (gradient 1). The integrity of the peptides was verified by MALDI-MS. Overall yields were 7 mg for G-12/Ser and 0.55 mg for G-11/Ser.

4.1.7. Construct G-12/Man. Successful synthesis of construct G-12/Man was achieved by manual coupling of two molar excess (0.16 g, 0.24 mmol, calculated to free amino groups on the resin) of *N*-Fmoc-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-L-serine onto the side chains of backbone lysines in construct A (made on 0.2 g of resin, 0.15 mmol g^{-1}). The amino acid was activated with an equivalent amount of HATU (calculated to the glycoamino acid) in the presence of 1 equiv. of diisopropylethylamine in 1.5 ml of DMF. The mixture was shaken for 3 days in a glass vial. After coupling and extensive washing with DMF, the final glycopeptide was cleaved off the resin with a mixture (5 ml) of TFA:water:thioanisole (90:5:5, v/v/v) for 4 h and precipitated into cold ether. Purification using preparative RP-HPLC (gradient 1) yielded 4.8 mg of the protected glycopeptide. MALDI-MS was used for the analysis of the protected product. Deacetylation and Fmoc removal were accomplished with a 30 min treatment employing 0.1M NaOH (1 ml per ~0.5 mg peptide). After neutralization with 0.2M HCl and further purification on preparative HPLC (gradient 1) 3.35 mg of deprotected glycopeptide G-12/Man was obtained. MALDI-MS was used for the final characterization.

4.1.8. Construct G-11/Man. Construct G-11/Man was successfully synthesized on a PS3 batch mixing synthesizer (Rainin Technologies, Woburn, MA) using double couplings (2×6 h) with *N*-Fmoc-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-L-serine onto construct B (made on 0.12 g of resin, 0.15 mmol g^{-1}) and HATU activation. While the first coupling employed a four molar excess (0.19 g, 0.29 mmol calculated to the number of free amino groups) of *N*-Fmoc-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-L-serine, the second used only 2 equiv. (0.095 g, 0.145 mmol). The glycopeptide was cleaved off the resin with a mixture of TFA:water:thioanisole (90:5:5, v/v/v) for 4 h and precipitated into cold ether and centrifuged. Because the very hydrophobic nature of the protected peptide prevented the RP-HPLC purification of the product (gradient 1), the Fmoc and acetyl protecting groups were removed from the crude glycopeptide with 0.1M NaOH as is described for G-12/Man. After neutralization with 0.2M HCl, and purification on preparative RP-HPLC (gradient 1) 0.03 mg of final glycopeptide G-11/Man was obtained. MALDI-MS was used for the characterization of the final product.

4.1.9. Conditions for manual coupling of Fmoc-Ser-(Ac_4-Man)-OPfp. Mannosylations of resin-bound constructs A (made on 0.30 g of resin, 0.15 mmol g^{-1}) and B (made on 0.12 g of resin, 0.15 mmol g^{-1}) were attempted with 2 equiv. of Fmoc-Ser(Ac_4-Man)-OPfp (0.29 g, 0.35 mmol for construct A and 0.125 g, 0.15 mmol for construct B in 2 and 1 ml of DMF, respectively) in the presence of 1 equiv. of diisopropylethylamine (and 1 equiv. of HOBt in the case of construct B). The mixtures were shaken for 2 days. Peptides were cleaved off the solid support with a mixture (2.5 ml per 0.1 g of resin) of TFA:water:thioanisole (90:5:5, v/v/v) for 4 h, precipitated in cold ether, centrifuged and filtered. Existence of the protected glycopeptides G-12/Man and G-11/Man was checked using analytical HPLC (gradient 1).

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