Journal of Peptide Science
J. Peptide Sci. **9**: 375–385 (2003)
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.463

JOURNAL OF PEPTIDE SCIENCE

Synthesis of a Functionalized High Affinity Mannose Receptor Ligand and its Application in the Construction of Peptide-, Polyamide- and PNA-Conjugates

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Received 28 January 2003
Accepted 5 February 2003

Abstract: The synthesis of a high affinity mannose receptor ligand, appropriately functionalized for chemoselective ligation with an antigen or DNA-binding moieties is described. By a combination of solid- and solution-phase chemistry a versatile synthesis of the target structure was accomplished. Examples of subsequent ligation reactions are described. Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: neoglycopeptide-conjugates; chemoselectivity; mannose receptor targeting

INTRODUCTION

A renewed appreciation of the crucial role played by dendritic cells (DC) in the activation of an immune response has prompted efforts to target antigens selectively to this cell population, for vaccination purposes [1]. One of the most promising ways for antigen internalization is to utilize the superior efficiency of mannose receptor (MR)-mediated uptake [2–4]. Moreover, in the context of DNA based vaccines, DC-selective delivery of nucleic acids through the MR would also be of considerable interest. Potent synthetic ligands for the MR have already been developed, taking advantage of the cluster effect [5,6], i.e. the simultaneous interaction of several, covalently linked carbohydrate units. In particular, Biessen *et al.* [7] designed the low molecular weight cluster mannoside **1**, which shows subnanomolar affinity for the MR (Figure 1).

We became interested in the possibility of labelling with the cluster mannoside **1**, in addition to peptide antigens, also plasmid DNA. To this end a flexible, convergent synthetic strategy was envisioned having at its centre a building block containing the cluster mannoside together with a long, flexible and water-soluble spacer, bearing at its end a functional group competent for chemoselective ligation. The ligation step would then take place with a suitably derivatized peptide/protein or a DNA-binding moiety, such as a bis-peptide nucleic acid (PNA) [8,9] or a pyrrole-imidazole polyamide [10,11]. The latter reactions would yield sequence-specific 'mini-vectors' for targeted gene delivery [12].

From the known ligation reactions, oxime formation between an aminoxy function on the cluster mannoside and a ketone or aldehyde on the reacting partner was selected. The latter functionality can be installed easily during solid-phase synthesis [13] or obtained by very mild periodate oxidation of an *N*-terminal serine residue [14,15]. The scope of oxime ligation is wider than the commonly used thiol-based ligation: the latter is not applicable to cysteine-containing biomolecules, and suffers, as

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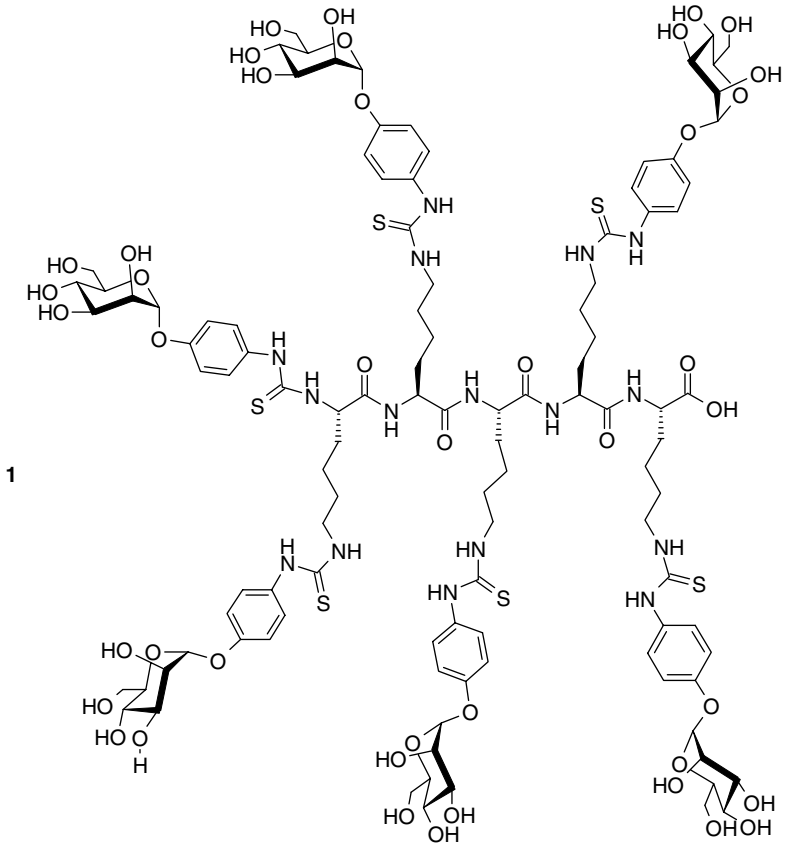

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Figure 1 Polylysine based cluster mannoside developed by Biessen *et al.* [7].

a side reaction, from the competing formation of disulfides under basic conditions. Finally, even for peptides whose sequence does not contain cysteines, the compatibility of oxime ligation with thiols allows for further chemoselective derivatization with, for example, a reporter group (biotin, fluorescein [16]).

The structure of the target cluster mannoside **2** is shown in Figure 2. Ligand **1** from Biessen *et al.* [7] is elongated from its *C*-terminus, which is most likely not important for the recognition by the MR. A long diamine spacer, containing a polyether unit to increase the water solubility, separates the aminoxy function from the ligand moiety. This should reduce any eventual interference of the latter attached biomolecules with the ligand recognition by the MR and also guarantee a good reactivity in the ligation reactions.

MATERIALS AND METHODS

Materials

1-*O*-(4-isothiocyanato-phenyl)-α-D-mannopyranose was obtained from Sigma-Aldrich S.r.l., Milan, Italy; PS-trityl chloride resin and amino acids were purchased from Calbiochem-Novabiochem AG, Läufelfingen, Switzerland; Fmoc-Linker AM-Champion resin was obtained from Biosearch Technologies Inc., Novato, CA, USA; carboxymethoxylamine hemihydrochloride and 4,7,10-trioxa-1,13-tridecanediamine from Fluka AG, Buchs, Switzerland. All other reagents and solvents used were of the highest commercially available grade and were used without further purification.

General

A Beckman System Gold® HPLC with Waters Symmetry® C_{18} columns was used: preparative, 100 × 19 mm, 7 μm; semipreparative, 150 × 7.8 mm, 7 μm; analytical, 150 mm × 4.6, 5 μm. The eluents used were water (0.1% TFA) and MeCN (0.1% TFA). Mass spectra were recorded using a Perkin Elmer API 100 instrument.

Resin 3

The procedure followed that described by Barlos *et al.* [17,18] Briefly: to 8 ml of a 5 : 1 mixture

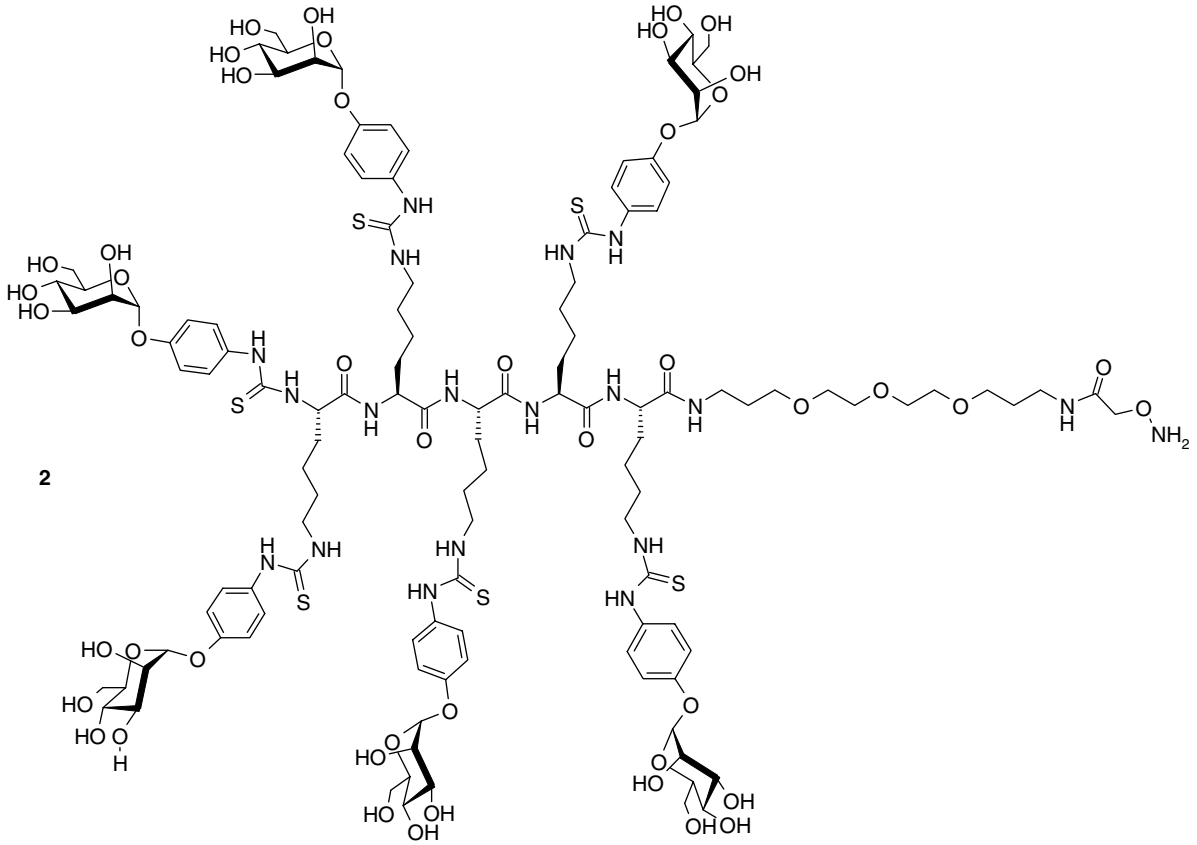


Figure 2 Target cluster mannoside **2**.

of anhydrous $CHCl_3$ and anhydrous acetonitrile were added carboxymethoxylamine hemihydrochloride (550 mg, 2.6 mmol), Et_3N (180 µl, 0.5 eq) and trimethylsilyl chloride (330 µl, 2.6 mmol). The resulting white suspension was heated to reflux for 2 h. The suspension was cooled to 0 °C and further Et_3N (600 µl, 4.3 mmol) was added and stirring was continued for 10 min at room temperature. Then 4-polystyryltriphenylmethyl chloride resin was added (1 g, 1.24 mmol) and the mixture was slowly magnetically stirred at room temperature for 6 h. The resin was then transferred into a syringe and sequentially washed with $CHCl_3$, MeOH, 5% aq. citric acid, H_2O, MeOH, THF and CH_2Cl_2. A negative Kaiser test indicated the absence of free primary amino groups. Resin **3** was dried for 1 h under high vacuum (dry weight: 1.083 g).

Resin **3** was evaluated by coupling with β-alanine-fluorenylmethylester: Resin **3** (25.65 mg) was swollen for 30 min in 300 µl DMF, drained and a solution of carbonyldiimidazole (63 mg, 0.39 mmol) in 300 µl DMF was added. The resin was shaken for 50 min, drained and washed with DMF. Then a solution of β-alanine-fluorenylmethylester (TFA salt, 38.0 mg, 0.099 mmol), HOBt (13.2 mg, 0.086 mmol) and DIPEA (37 µl, 2.4 eq respective amino acid) in DMF (300 µl) was added. The resin was shaken for 1 h, drained and washed with DMF and CH_2Cl_2. After 1 h under high vacuum the loading was determined by fluorenylmethylidene quantitation [19] and found to be 0.34 mmol/g.

Resin 4

The procedure followed that described by Rose *et al.* [20] Briefly: resin **3** (1.083 g) was preswollen in DMF, drained and a solution of carbonyldiimidazole in DMF (1.30 g, 8 mmol in 8 ml) was added. The resin was shaken for 30 min, drained and washed with DMF. Then a solution of HOBt in a 1 : 1 mixture of DMF and 4,7,10-trioxa-1,13-tridecanediamine (0.5 M, 8 ml) was added. After initial short vortexing the resin was shaken for 1 h, then drained and washed with DMF and CH_2Cl_2. The Kaiser test showed an intense blue colour, indicative of free primary amino groups. Resin **4** was dried under high vacuum for 1 h (dry weight: 1.27 g).

Peptide 5

From resin **4** the synthesis of peptide **5** was carried out by standard Fmoc/tert-butyl chemistry. Couplings were performed using N_α-Fmoc-N_ε-Boc-L-lysine/PyBOP/HOBt/DIPEA (molar ratio 1 : 1 : 1 : 2) in DMF with a 3-fold molar excess over resin amino groups (coupling time 1 h). N_α-Deprotection was achieved by treatment with 20% piperidine in DMF for 20 min. The loading after the coupling of the first lysine residue was 0.22 mmol/g (fluorenylmethylidene quantitation). Cleavage and Boc-deprotection was achieved by treatment with reagent B (TFA 87.5%, water 5%, phenol 5%, TIPS 2.5%, 25 ml) for 2 h. The crude deprotected peptide **5** was precipitated with cold MTBE and purified by preparative RP-HPLC. After lyophilization 375 mg of **5** as a fluffy white material was obtained (0.22 mmol, MW calculated with 7 × TFA, 68% yield with respect to the loading determined after the first coupling, 94% purity by HPLC, A_{214}). LRMS: calcd for $C_{42}H_{87}N_{13}O_{10}$ 933.7, found 934.0.

Peptide 6

To a solution of peptide **5** (188 mg, 0.107 mmol, MW calculated as with 7 × TFA) in 5 ml acetic acid was added a solution of triphenylmethylcarbinol (93 mg, 0.36 mmol) in 2 ml CH_2Cl_2. To the resulting clear solution was added dropwise under stirring $BF_3{\cdot}OEt_2$ (44 µl, 0.36 mmol). The mixture was stirred for 100 min at room temperature and then injected into cold Et_2O. The precipitate was dissolved in water and an undissolved residue was removed by filtration. The crude peptide **6** was purified by preparative RP-HPLC. After lyophilization 160 mg of **6** as a fluffy white material was obtained (0.086 mmol, 80% yield, MW calculated with 6 × TFA, 91% purity by HPLC, A_{214}). LRMS: calcd for $C_{61}H_{101}N_{13}O_{10}$ 1175.8, found 1176.0.

Mannoside Cluster 2

To a solution of peptide **6** (63 mg, 35 µmol) in a mixture of 2 ml DMF and 2 ml 0.1 M $NaHCO_3$ was added 1-*O*-(4-isothiocyanato-phenyl)-α-D-mannopyranose (**6b**, 79 mg, 250 µmol). The reaction progress was monitored by analytical HPLC. After 18 h further **6b** (54 mg, 170 µmol) and $NaHCO_3$ (20 mg) were added. After 22 h the reaction was complete and 10 ml H_2O was added to the mixture. The resulting white precipitate was filtered off and resuspended in a mixture of 2 ml water and 100 µl isopropylamine. After 10 min stirring at room temperature 2 ml ethanol was added and the mixture was concentrated to dryness under reduced pressure. The dry precipitate was triturated with 2 × 5 ml of Et_2O and left under high vacuum for 2 h.

The crude reaction product **7** was deprotected by treatment with 3% TFA, 2% TIPS in CH_2Cl_2 (20 ml) at room temperature for 10 min. The solution was poured into 100 ml MTBE and the resulting precipitate was washed with 50 ml MTBE. The precipitate was lyophilized from a mixture of water/acetonitrile (7 : 3), to afford 66 mg of crude **2**, which was purified by preparative RP-HPLC (eluents without TFA). After lyophilization of the pooled product fractions 29.3 mg of **2** as a fluffy white material was obtained (30% yield from **6**, 96% purity by HPLC, A_{214}). LRMS: calcd for $C_{120}H_{177}N_{19}O_{46}S_6$ 2813.1, found 2813.0.

Bis-PNA 8

The bis-PNA **8** 5′-K-O-TCTCTCTC-O-O-O-JTJTJTJT-3′ (Figure 4) was synthesized manually on a MBHA-resin as described [21]. LRMS: calcd for $C_{204}H_{282}N_{78}O_{73}$ 4994.1, found 4994.0.

Polyamide 9

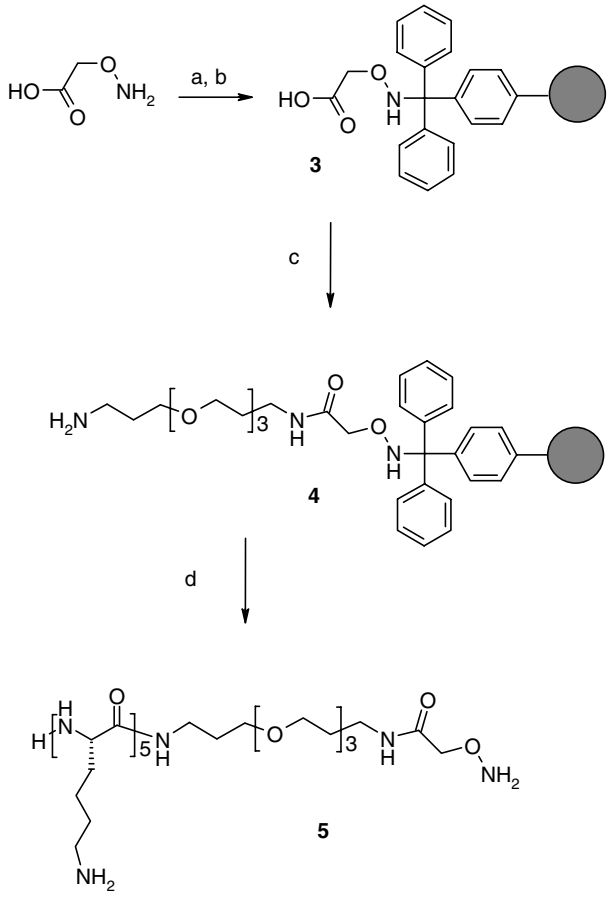
The precursor of polyamide **9**, having a free amino-terminus was synthesized as described earlier [22]. The 5-oxo-hexanoyl residue was installed via reaction with the active ester *N*-succinimidyl-5-oxo-hexanoic acid [23]: To a solution of polyamide with free *N*-terminus (4.8 mg, 4.1 µmol) in a mixture of 50 µl DMF and 25 µl DIPEA was added of *N*-succinimidyl-5-oxo-hexanoic acid (20 mg, 88 µmol). The mixture was left standing at room temperature for 25 min and was then neutralized with 25 µl of acetic acid. After purification by preparative RP-HPLC 2.2 mg (1.9 µmol, 46%) of polyamide **9** were obtained. LRMS: calcd for $C_{55}H_{73}N_{17}O_{10}$ 1131.6, found 1131.6.

Peptide 10

The peptide with an *N*-terminal serine residue was assembled by machine-assisted Fmoc/tert-butyl chemistry, on Fmoc-Linker AM-Champion resin (1% cross-linked, PEG-PS, 0.34 mmol/g), using HBTU/DIPEA (1/2) activation, a 5-fold excess of acylants over the resin amino groups, and coupling times of 60 min. The peptide was cleaved and deprotected by treating the resin with reagent B (TFA 87.5%, water 5%, phenol 5%, TIPS 2.5%,

25 ml) for 80 min. The resin was filtered and rinsed with TFA, the TFA solution was concentrated and added dropwise to cold MTBE. The formed precipitate was collected by centrifugation. The obtained pellet was washed twice by resuspension in MTBE and centrifugation. The crude lyophilized product was purified by preparative RP-HPLC. LRMS: calcd for $C_{147}H_{215}N_{41}O_{41}S_3$ 3305.8; found 3306.2.

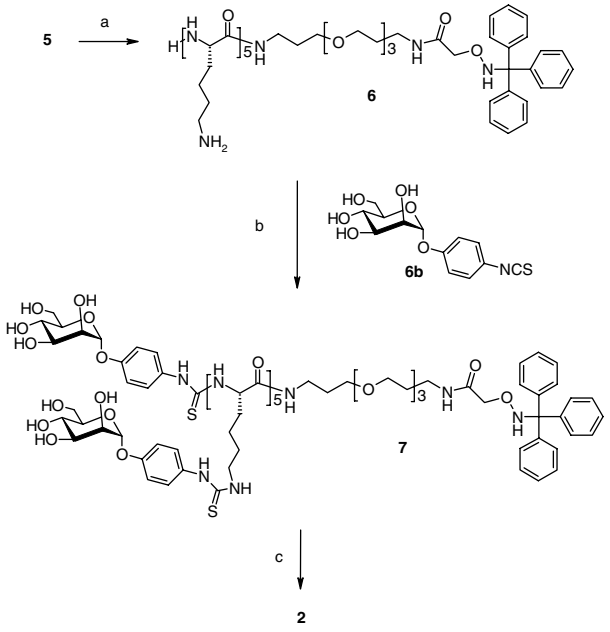
Oxidation of the *N*-terminal Ser to aldehyde [14,15,24]: In order to prevent the disulfide formation between the Cys residues the peptide was refolded in a typical zinc-finger folding by coordination of the Cys and His residues to a zinc ion at neutral pH: 12 mg of peptide was dissolved in 0.2 mM $ZnCl_2$ at a concentration of 0.2 mg/ml, then concentrated to 1 mg/ml and the pH was adjusted to 6.3 with 0.2 M Na_2HPO_4. Then 100 eq (57 mg dissolved in 1 ml of water) of methionine and 2 eq of sodium periodate (330 µl of a 5 mg/ml solution in water) were added to the peptide solution and left stirring at room temperature. After 15 min another aliquot of 100 eq of methionine was added, the solution was acidified with TFA (0.1%) and the product was purified by RP-HPLC to obtain 7 mg **10** (7 mg, 58%). LRMS: calcd for $C_{146}H_{210}N_{40}O_{41}S_3$ 3275.0, found 3274.6.



Scheme 1 Solid-supported synthesis of aminoxy-functionalized polylysine scaffold **5**. (a) Me_3SiCl, TEA, $CHCl_3$, MeCN, reflux, 2 h; (b) 1. Et_3N, 0 °C, 10 min, 2. PS-trityl-Cl, rt, 6 h; (c) 1. Carbonyldiimidazole, DMF, rt, 50 min, 2. diamine, DMF, HOBt, rt, 1 h; (d) Couplings: Fmoc-Lys (Boc)-OH, PyBOP, HOBt, DIPEA, rt, 1 h; Deprotections: Piperidine/DMF 20 : 80, rt, 20 min; Cleavage: reagent B, rt, 2 h.

Conjugate 11

To a solution of **8** (1.6 mg, 0.27 µmol) in acetate buffer pH 4.0 (100 µM) was added **2** (2.8 mg, ca. 4 eq resp. **8**). The solution was left standing at room temperature and the reaction progress was monitored by RP-HPLC. After 2 h the reaction was complete. Water was added (900 µl) and the ligation product **11** was purified by semipreparative RP-HPLC. After lyophilization 1.4 mg (0.178 µmol, 67% yield, 96% purity by HPLC, A_{214}) of **11** as a fluffy white material was obtained. LRMS: calcd for $C_{324}H_{457}N_{97}O_{118}S_6$ 7791.1, found 7789.0.



Scheme 2 Solution phase synthetic sequence from unprotected polylysine scaffold **5** to target structure **2**. (a) triphenylmethanol, $BF_3{\cdot}OEt_2$, HOAc, CH_2Cl_2, rt, 100 min; (b) **6b** (12 eq), 0.1 M $NaHCO_3$, DMF, 22 h; (c) CH_2Cl_2, 3% TFA, 2% TIPS, rt, 10 min.

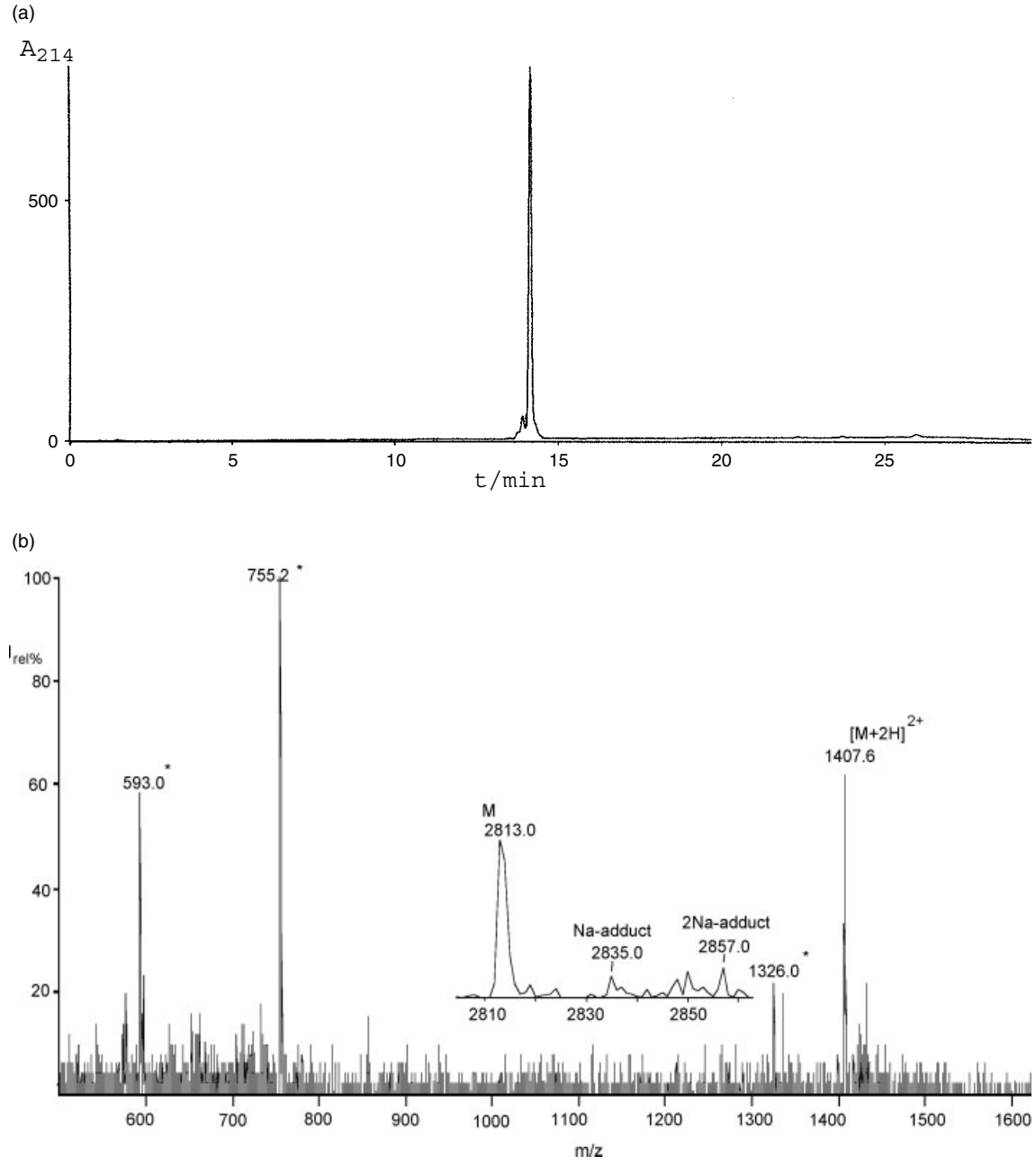


Figure 3 (a) HPLC chromatogram of purified compound **2**. Conditions: Waters Symmetry® C_{18} (5 µm, 4.6 × 150 mm). Flow rate 1 ml/min. Eluents A: H_2O, 1% TFA; B: MeCN, 1% TFA. Gradient: 5%–45% B over 25 min. (b) Positive MS of **2** with calculated true mass scale of molecular ion, ∗ identified fragments of **2**.

Conjugate 12

To a solution of **9** (0.4 mg, 0.32 µmol) in 20 µl DMF and 20 µl acetate buffer (0.1 M, pH 4.0) was added cluster mannoside **2** (1.7 mg, 1.9 eq). The solution was left standing at room temperature for 2 h. The product was purified by semipreparative RP-HPLC. After lyophilization 1.0 mg (0.247 µmol, 77% yield, 96% purity by HPLC, A_{214}) of conjugate **12** as a white fluffy material was obtained. LRMS: calcd for $C_{175}H_{248}N_{36}O_{55}S_6$ 3927.6, found 3928.2.

Conjugate 13

To a solution of **10** (8.9 mg, 2.18 µmol) in 150 µl acetate buffer (0.1 M, pH 4.0) was added cluster

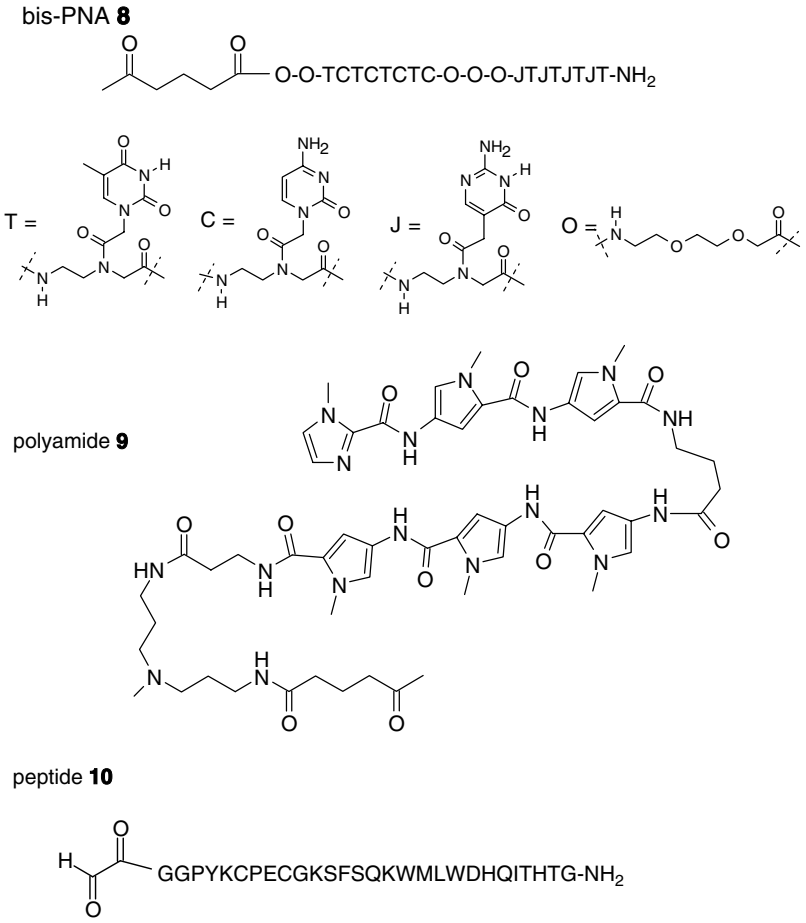
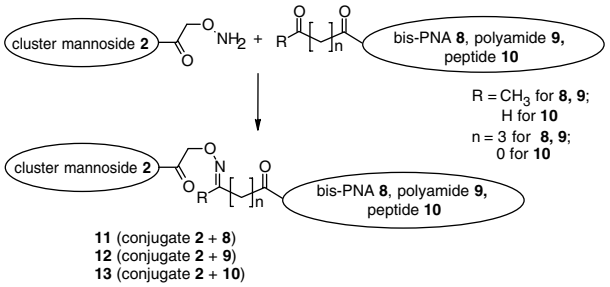


Figure 4 Molecules used in ligation reactions with **2**.

mannoside **2** (7.9 mg, 1.2 eq). The solution was left standing at room temperature for 90 min. The product was purified by semipreparative RP-HPLC. After lyophilization 3.27 mg (0.48 μmol, 22% yield, 98% purity by HPLC, A_{214}) of conjugate **13** as a white fluffy material was obtained. LRMS: calcd for $C_{266}H_{385}N_{59}O_{86}S_9$ 6071.5, found 6072.0.



Scheme 3 Ligation reactions. **2** with **8**: 0.1 M acetate, pH 4.0, 4 eq of **2**, 2 h. **2** with **9**: 1 : 1 (v/v) DMF/0.1 M acetate pH 4.0, 1.9 eq of **2**, 2 h. **2** with **10**: 0.1 M acetate pH 4.0, 1.2 eq of **2**, 1.5 h.

RESULTS AND DISCUSSION

When planning the synthesis of target structure **2** it was anticipated that it would be difficult to obtain it by a coupling reaction at the free carboxyl group of **1** with a suitably protected linker moiety. The crowded periphery of **1** would probably reduce the accessibility of the carboxyl group and lower its reactivity. An eventually necessary protection of the free hydroxyl groups would even increase the sterical shielding around the carboxyl group. It was therefore decided to build up target structure **2** on a solid support, starting from resin-attached aminoxyacetic acid. For the attachment of the aminoxyl group on a solid support our choice fell on the commercially available PS-trityl chloride resin, since the trityl linker would allow for a mild

acidic cleavage of **2** from the resin without affecting the mannose residues. As described for α-amino acids [18], after a transient protection of the carboxyl group as a TMS ester, aminoxyacetic acid could be loaded onto the PS-trityl resin in a convenient one-pot reaction (Scheme 1). A satisfactory loading of 0.34 mmol/g was obtained, which was determined by fluorenylmethylidene quantitation [19] after coupling of a small sample of resin with β-alanine-fluorenylmethyl ester. As the next step, the linker element 4,7,10-trioxatridecanediamine was easily introduced without the need for a cumbersome mono amino-protection, by using a large excess of diamine, as described by Rose *et al.* [20]

An initial attempt to assemble the cluster mannoside by using lysines already mannosylated at their ε-amino group (with sugar hydroxyl groups protected as acetates) turned out to be problematic. Coupling times increased with increasing peptide chain length and the coupling of the final amino acid could not be completed. This was attributed to increasing steric hindrance at the free α-amino group with increasing length of the cluster mannoside. Similarly unsuccessful was the attempt to introduce the mannose residues on the amino-deprotected lysine-scaffold, still resin-bound, via thiourea formation, again probably because of insufficient accessibility of the free amino groups.

Therefore the idea was abandoned of completing the synthesis of our target molecule attached on the resin and we proceeded to intermediate **5**, the unprotected lysine scaffold, containing the linker with the aminoxyacetyl residue (Scheme 1).

At this point a key requirement of the synthesis had become a procedure to selectively protect the aminoxy function, leaving unaffected the primary amino groups. Moreover, the protecting group should be easily removable in the presence of unprotected sugar residues. Attempts to find conditions for the cleavage of oximes, which might be compatible with the sugar residues, were not successful. Known procedures for oxime cleavage depend on the use of strongly acidic conditions or Lewis acids in the presence of excess ketone [25,26]. In our search for alternatives to the oxime formation/cleavage it was finally found that a selective protection of the aminoxy function can be achieved by a modified procedure for the *S*-tritylation of cysteine [27]. The Lewis acid mediated reaction takes place under mild acidic conditions ($HOAc/CH_2Cl_2$ as solvent) where the primary amino groups of the lysine residues should be protonated, while the aminoxy function retains a good reactivity. After optimization of the reaction conditions the aminoxy-protected peptide **6** was obtained in 80% yield (Scheme 2).

With the intermediate **6** obtained the mannose residues were then installed on the remaining free amino groups via formation of thioureas (Scheme 2). In solution the build-up of the mannose periphery proceeded smoothly and was complete after 22 h. After a short workup the trityl-protected precursor of **2** (intermediate **7**) was then subjected to the deprotection procedure. Treatment with 3% TFA/2% TIPS in CH_2Cl_2 for 10 min was sufficient for a complete deprotection. As anticipated, the mild acidic conditions left the acid-sensitive glycoside moieties intact. The overall yield of **2** (from **6**) was 30% after HPLC-purification. The HPLC-chromatogram and the mass spectrum of the purified cluster mannoside **2** are shown in Figure 3.

To finalize the synthesis of the gene delivery 'mini-vectors' a ligation was performed of cluster mannoside **2** with two known, high-affinity, sequence-specific DNA ligands. The first one was the well-established bis-PNA **8** (Figure 4, Scheme 3), consisting of a structure developed by Zelphati

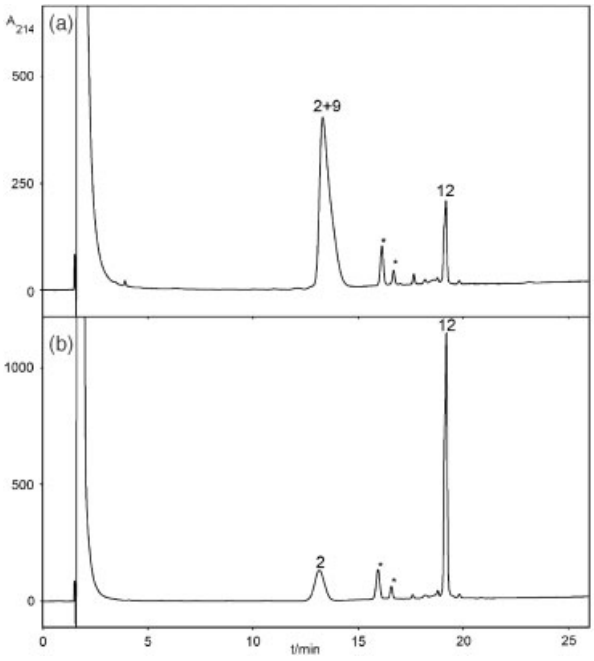


Figure 5 HPLC chromatograms of ligation of cluster mannoside **2** with polyamide **9**. (a) after 10 min, (b) after 2 h, ∗ unreactive degradation products of **2**. Conditions: Waters Symmetry® C_{18} (5 μm, 4.6 × 150 mm). Flow rate 1 ml min^{-1}. Eluents A: H_2O, 1% TFA; B: MeCN, 1% TFA. Gradient: 20% B from 0 to 11 min, then 20%–45% B over 15 min.

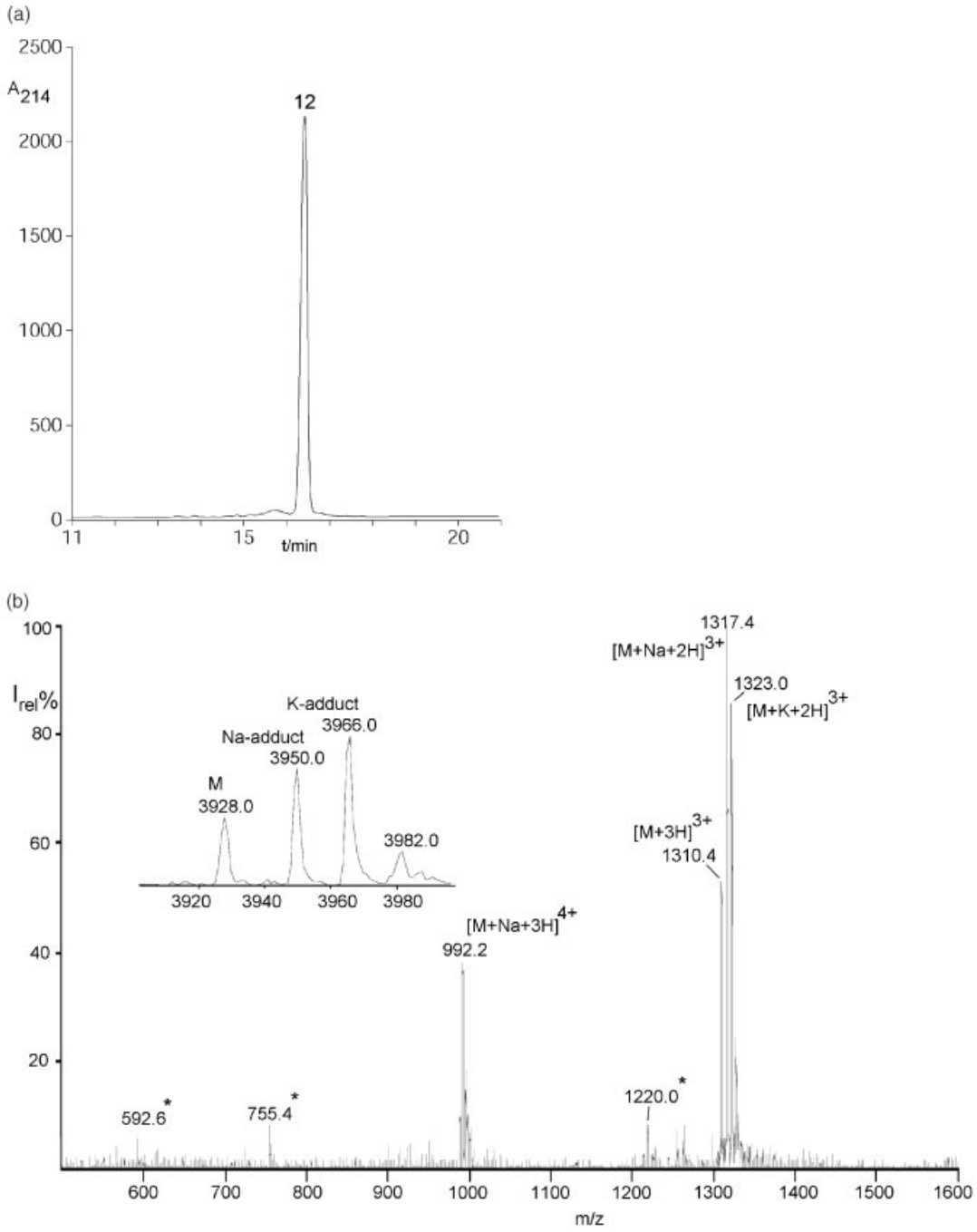


Figure 6 (a) HPLC chromatogram of purified compound **12**. Conditions: Waters Symmetry® C_{18} (5 μm, 4.6 × 150 mm). Flow rate 1 ml min^{-1}. Eluents A: H_2O, 1% TFA; B: MeCN, 1% TFA. Gradient: 25%–45%B over 20 min. (b) Positive MS of **12** with calculated true mass scale of molecular ion, ∗ identified fragments of **12**.

et al. [8], which was derivatized with 5-oxo-hexanoic acid at its 5′ terminus, for the ligation via ketoxime formation [13,28]. The second one was the pyrrole-imidazole polyamide **9** (Figure 4, Scheme 3), developed by Dervan *et al.* [10,11], again derivatized at its *N*-terminus with 5-oxo-hexanoic acid [28]. The ligation-reaction with **8** was carried out in 0.1 M acetate buffer at pH 4.0, the reaction with **9** in a 1 : 1 mixture of DMF and acetate buffer, pH 4.0. Both reactions were complete after about 2 h, confirming

the good reactivity of the aminoxyl group of the cluster mannoside **2** in the ketoxime-ligation. As an example, the time course of the ligation of **2** with **9** is shown in Figure 5, while Figure 6 shows the HPLC chromatogram and the mass spectrum of purified conjugate **12**.

The usefulness of **2** in the preparation of MR-targeted immunogens from cysteine-containing peptides is demonstrated by the reaction with peptide **10** (Figure 4, Scheme 3). The 29-amino acid peptide contains two cysteine residues, and an *N*-terminal serine, which was added to the sequence as the precursor for the aldehyde function. The *N*-terminal aldehyde was obtained in high yield by periodate oxidation [14,15]. The pure ligation product **13** was obtained, although with an overall yield lower than for the ligations with **8** and **9** (22% for **13**; 67%, 77% for **11, 12** respectively).

CONCLUSIONS

In summary, in this study a straightforward synthesis of the MR-targeting cluster mannoside **2** was achieved by an appropriate combination of solid and solution phase chemistry. The usefulness of the trityl group as a linker for aminoxyl functions on solid support as well as a selective protecting group for this functionality in solution phase chemistry is shown, which should widen the scope of its applicability in chemoselective reactions. Moreover the synthesis requires only commercially available starting materials.

The versatility of the cluster mannoside **2** was shown by three ligation reactions with two different DNA-binding moieties and a cysteine-containing peptide. Overall this strategy lends itself to the repetitive approach, which is desirable to fine-tune the immunological properties of vaccine candidates. Importantly, it is compatible also with thiol-containing biomolecules.

Acknowledgements

The authors wish to thank Philippe Neuner for the synthesis of the Bis-PNA **8**, Barbara Gatti for assistance with peptide synthesis, Fabio Bonelli, Anna Alfieri and Laura Orsatti for analytical mass spectrometry, Gennaro Ciliberto and Riccardo Cortese for useful criticism throughout the work.

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