Organic & Biomolecular Chemistry

Cite this: *Org. Biomol. Chem.*, 2012, **10**, 3116

www.rsc.org/obc

PAPER

Reverse thioether ligation route to multimeric peptide antigens†

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Received 29th October 2011, Accepted 8th February 2012
DOI: 10.1039/c2ob06819b

Multimeric presentation, a rather effective way of enhancing peptide immunogenicity, is best exemplified by MAP (multiple antigenic peptide) dendrimers consisting of a branched Lys core on which several copies of the peptide epitope are displayed. While accessible by solid-phase synthesis, MAPs can also be conveniently made in solution, *e.g.*, by linking the epitope (*N*-acetylated and with a C-terminal Cys) through a thioether bond onto the α and ε (haloacetyl-activated) positions of the Lys core. We now report the reverse version of this approach, whereby a chloroacetyl-derivatised epitope is tethered to a thiol-functionalised form of a Lys dendron core. This convergent approach can be carried out either in solution or in the solid phase and is advantageous because (i) *in situ* tris(2-carboxyethyl)phosphine (TCEP)-mediated reduction of disulfide bonds maintains the thiol platform reactive throughout the ligation process; (ii) the low amounts of TCEP used pose minimal risk to chloroacetyl groups in the peptide, resulting in (iii) significantly reduced byproduct formation, hence cleaner products. For the solid phase version of the method, an optimised procedure has been devised to convert the Lys core into a tetrathiol dendron.

Introduction

Although conjugation to carrier proteins remains the most popular strategy for presentation of short peptide epitopes to the immune system,[1] carrier-bound peptides are far from ideal in terms of analytical documentation and synthetic reproducibility. On the analytical side, amino acid analysis, the only general method for peptide–protein conjugate documentation, gives only approximate and often unreliable epitope-to-carrier ratios, particularly with large carriers such as the much-favored keyhole limpet hemocyanin (KLH, with MWs diversely reported in the 2000–7000 kDa range). In addition, the variability inherent to even the best-defined conjugation chemistries tends to create difficulties for batch standardisation. All this is compounded by the fact that, in antisera raised against conjugates, anti-carrier antibodies tend to predominate over anti-peptide ones, which often require additional affinity enrichment steps.

In view of all these difficulties, different approaches have been proposed as alternatives for enhancing the immunogenicity of linear peptides,[2–4] of which multimerisation[5] is recognised as rather effective. In particular, the multiple antigenic peptide (MAP) constructions pioneered by Tam[6] and consisting of a Lys dendron core to which 2, 4 or 8 epitopes are attached (Fig. 1), have received the most attention. MAPs have an advantage over peptide–protein conjugates in that they have chemically defined structures, readily accessible by peptide synthesis methods. While originally available by solid-phase peptide synthesis (SPPS) protocols,[6] MAPs have also been made in solution by various convergent approaches such as thioether-,[7] oxime-,[8] disulfide-,[9] thioester-,[10] thiazolidine-,[11] or hydrazone[12]-based ligations, as well as by native chemical ligation.[13] The relative merits of either ligation or SPPS strategies have not been sufficiently assessed in comparative studies and perhaps can be only fairly gauged on a case-by-case basis.[14,15] Still, for the SPPS approach one must accept that, as the epitopes built on the dendron core increase in size, the likelihood of hard-to-predict and/or -avoid episodes such as chain aggregation or deletion also increases and becomes inconveniently amplified by the simultaneous multiple nature of the assembly process, thereby rising the risk of unmanageably heterogeneous end products. Although some of these difficulties can be partially mitigated by optimised, MAP-dedicated SPPS methodology,[16] the convergent approach whereby an appropriately functionalised dendron core and a peptide epitope, both conveniently prepurified, are chemoselectively conjugated in solution, remains an attractive and versatile option. Among the aforesaid chemistries used to this end, thioether ligation stands out for its simplicity, good yields and the metabolical stability of its products (compared, *e.g.*, to the acid lability of oxime or hydrazone ligation products).[17] The standard approach to thioether ligation[18] involves (Fig. 1A) haloacetyl (XAc, X = Cl or Br) α- and ε-derivatisation of the Lys core followed by nucleophilic substitution of the halogen by

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†Electronic supplementary information (ESI) available: HPLC traces of Table 1 compounds. See DOI: 10.1039/c2ob06819b

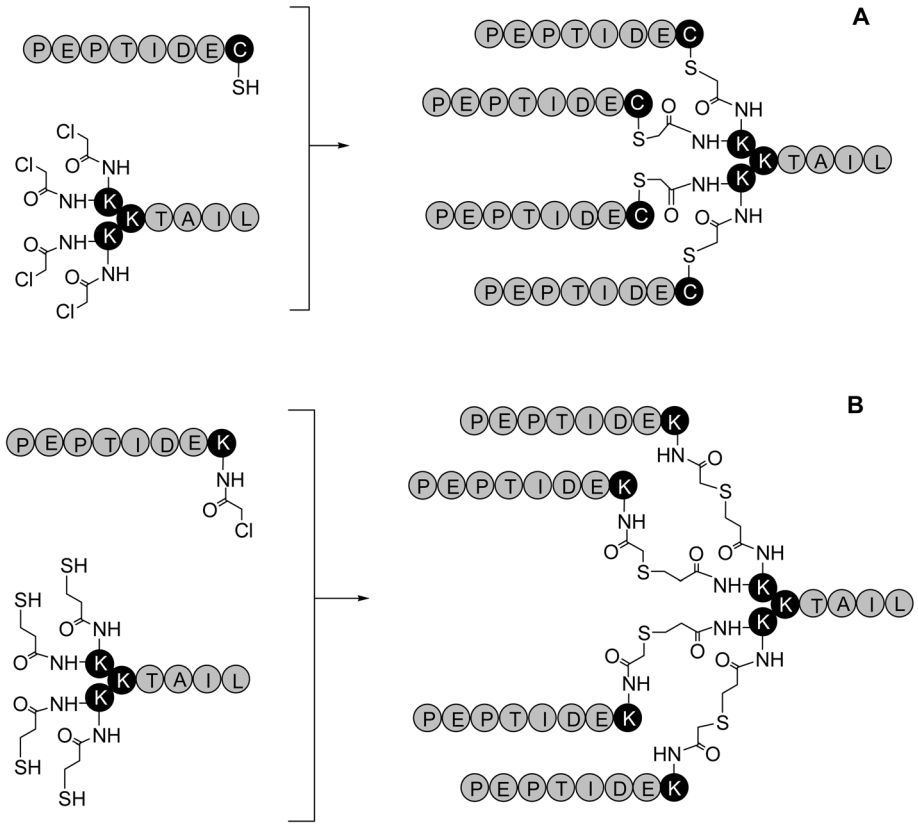

Fig. 1 Thioether-based approaches to multimeric peptides. In standard approach (A), the peptide reacts through its Cys thiol with a ClAc-functionalised poly-Lys dendron; in the reverse approach (B), the dendron core is functionalised as a thiol and reacted with ClAc-peptide.

the thiol group of an N- or C-terminal Cys-containing peptide. Although successfully used,[7,19,20] the method has the serious drawback that, at pH ≥ 7 required for thioether formation, dimerisation of the (free thiol) peptide may prevail over thioether synthesis,[14,16] especially since the only way to overcome sluggish ligation is to use the Cys-peptide in large amounts, much of which are unproductively turned into dimer. To avoid such waste, we have explored the possibility of conducting thioether bond formation in the reversed mode (Fig. 1B), by derivatising the Lys dendron core with thiol groups and reacting it with XAc-functionalised peptide epitopes. While conjugation of thiol-functionalised Lys-based dendrons with peptide epitopes *via* disulfide bonds has been described,[9,21] it has not enjoyed wide acceptance, possibly because the complexity of thiol-disulfide exchange reactions made the approach difficult to control. We have revisited the strategy with a substantial improvement, namely replacing disulfide- by thioether-based ligation, which to the best of our knowledge has not been attempted before in the reverse format proposed here, either in solution or in the solid phase.

Results and discussion

1. Reverse thioether ligation in solution

In our novel approach to thioether ligation a polythiol dendron core reacts with an XAc-containing peptide, opposite to the conventional practice[18] (Fig. 1). The feasibility of this approach was first explored in solution by a simple model consisting of ClAc-peptide **1** and tetravalent thiol core **2** (Table 1), both designed with a view to optimal synthesis, solubility and unambiguous product identification by MALDI TOF-MS.

Peptides such as **1**, with XAc groups at predefined positions, are conveniently made by Fmoc SPPS by introducing Mmt-protected Lys residues at the specified points,[22,23] often the N- or the C-terminus. The Mmt group is chemoselectively removed on-resin at the end of the synthesis (with prior N^{α}-Fmoc deprotection and acetylation) by mild acid treatment (1% TFA) that leaves unaltered other protecting groups on the resin-bound peptide. After Mmt deprotection, an haloacetic acid (XAcOH) is coupled to the free ε-amino group before final deprotection and cleavage of the peptide-resin. Of the two XAc groups usually employed in thioether ligations, BrAc is more reactive toward nucleophiles and potentially more efficient, but also undesirably reactive with water, not only during the ligation steps but even with trace H_2O in freeze-dried material, hence ClAc was preferred. Derivatisation of the branched Lys core to give tetrathiol peptide **2** was performed in the solid phase by DIPCI-mediated acylation with 3-tritylmercaptopropionic acid (Trt-Mpa). Advantageously over other Mpa derivatives, Trt-Mpa is an odourless, easy to handle solid, and its Trt protecting group is removed at the final deprotection and cleavage stage, so that dendron **2** is directly obtained in free thiol form.

The advantages of the reverse over the standard approach become further evident in the actual thioether ligation step,

Table 1 Linear and dendrimeric peptides used in this study

	Structure[a]	Mass (Da)		
		Calcd[b]	Found[c]	HPLC[d]
1	KSHAGK(ClAc)	701.37	702.42	5.6 (0–20)
2	$[(HSCH_2CH_2CO)_2K]_2KRRGG$	1179.71	1180.43	10.3 (0–30)
3[e]	$[(KSHAGK)_2K]_2KRRGG$	3842.39	3843.01	10.9 (0–20)
4	Ac-EVETPTRNEWESK(ClAc)	1721.73	1721.30	5.5 (10–60)
5	$(HSCH_2CH_2CO)_2KKK\beta A$	648.35	649.14	5.8 (0–30)
6	$[(HSCH_2CH_2CO)_2K]_2KKK\beta A$	1080.53	1080.99	7.1 (5–40)
7[f]	$(Ac\text{-}EVETPTRNEWESK)_2KKK\beta A$	4019.85	4018.13	12.3 (0–30)
8[g]	$[(Ac\text{-}EVETPTRNEWESK)_2K]_2KKK\beta A$	7823.54	7825[h]	9.1 (15–30)

[a] Sequences written from N- to C-terminus; branching in dendrimeric peptides **3**, **7** and **8** as in Fig. 1, panel B. [b] Monoisotopic mass. [c] MALDI-TOF MS reflector mode, unless indicated. [d] Retention time, in minutes; gradient (% of solvent B into A over 15 min) in parenthesis. [e] Reverse ligation of **1** + **2**. [f] Reverse ligation of **4** + **5**. [g] Reverse ligation of **4** + **6**. [h] Determined by LC-MS.

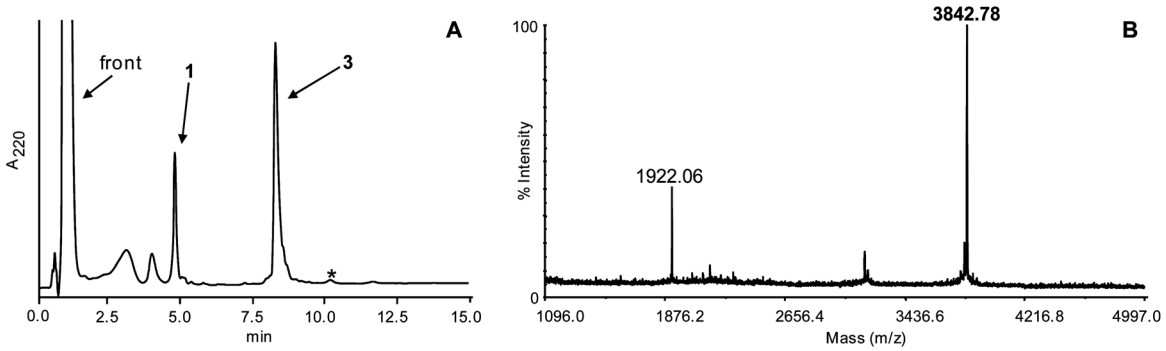

Fig. 2 (A) Analytical HPLC of the reverse thioether ligation leading to **3** (t = 24 h). Peak numbers as in Table 1. ClAc peptide **1** used in 8-fold molar excess (2 eq. per branch). The elution position of (used up) tetrathiol **2** is marked with an asterisk. The broad peaks eluting before 5 min correspond to non-peptide, low MW products, possibly TCEP-related. (B) MALDI-TOF MS of purified **3**; peaks for single and double-charged ions are observed.

illustrated in Fig. 2 for a ClAc peptide **1** and polythiol **2**. In the standard method, in addition to the aforesaid waste of thiol-functionalised peptide by dimerisation, slow rates and side reactions compound to give low yields of target (*e.g.*, four-branched) product contaminated by lower-order (three-, two-branched) byproducts that require painstaking purification, even when high excesses (typically 12–16-fold, 3–4 eq. per branch) of thiol component are used.[16] In contrast, dimerisation is not an issue in the reverse approach, hence reactions can be driven to completion more economically, 2 eq. per branch of ClAc peptide (**1** or **4** in Table 1) typically sufficing for a homogeneous product to be obtained. The key to these advantages is the supplementing of the reaction mixture with TCEP, which *in situ* reduces any disulfides,[24,25] hence ensuring a permanently reactive thiol component (Fig. 3A). Moreover, the amounts of TCEP required to this end are modest, hence the risk of nucleophilic displacement of the chlorine of the ClAc group[26] by TCEP – a serious drawback in the conventional approach (Fig. 3B) – is averted, and a cleaner, easily purifiable product results (Fig. 3C).

After the proof-of-concept trial (Fig. 2), the efficacy of the reverse approach has been verified for more relevant peptide epitopes to be included in candidate vaccines. This is illustrated here for bivalent (**7**, Fig. 3C) and tetravalent (**8**, Fig. 4B and C) presentations of the influenza virus M2e epitope (Table 1).

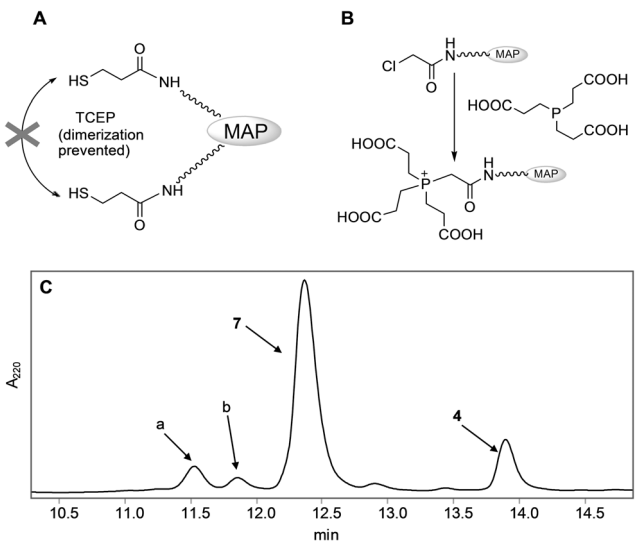

Fig. 3 (A) In the reverse approach, *in situ* TCEP reduction prevents dimerisation; in contrast, in the standard approach (B) TCEP blocks ClAc reactive sites in the dendron core. (C) HPLC analysis of the **4** + **5** ligation to bivalent dendron **7** illustrates advantages of the reverse approach; in order of elution: (a) monovalent product, (b) tris-carboxyethylphosphonium derivative of **4**, resulting from replacement of chlorine in ClAc by TCEP; target dendrimer **7**, and unreacted ClAc peptide **4**.

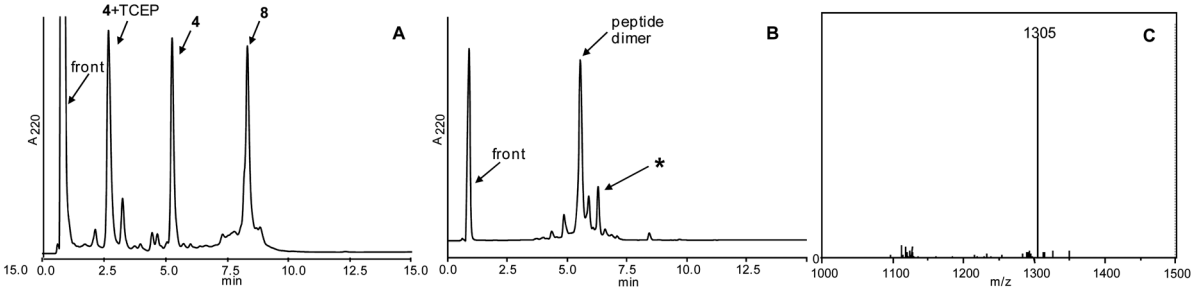

Fig. 4 Thioether-based approaches to tetravalent dendrimers. (A) HPLC analysis shows a clean reaction mixture (t = 24 h) for construct **8** by the reverse approach. (B) In contrast, an **8**-like product made by the standard approach shows (t = 24 h) very low levels of the target dendrimer (*) and extensive dimerisation of the thiol peptide (see ref. 16 for more details). (C) Mass spectrum (LC-MS) of purified **8**; the $[M + 6H^+]/6$ peak is shown.

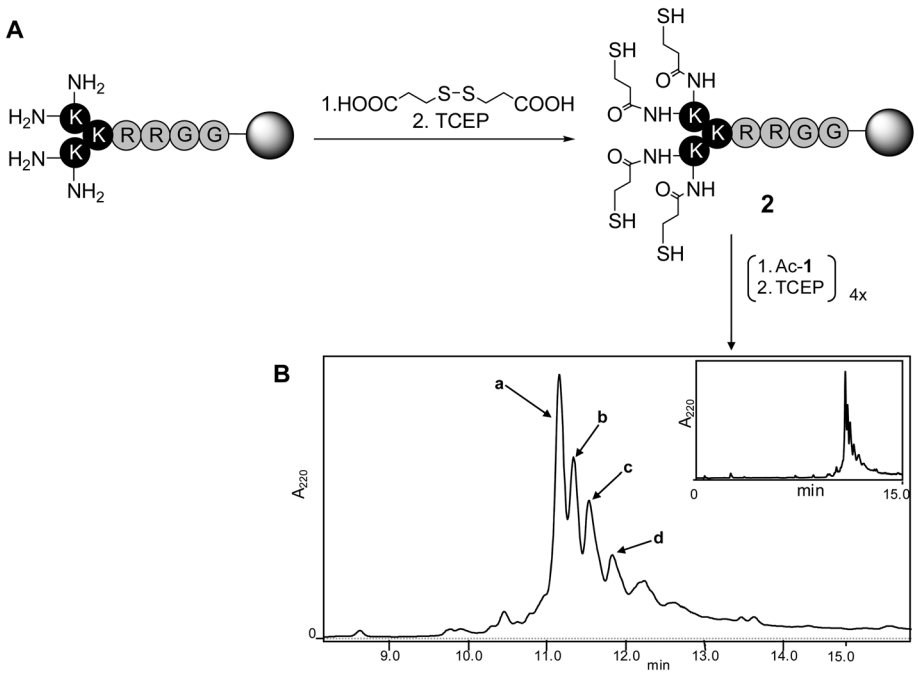

Fig. 5 (A) Thiol functionalisation of dendron core **2** for solid phase-adapted reverse thioether ligation with acetylated **1**, leading to a **3**-like product. (B) HPLC analysis of the ligation product upon cleavage from resin: (a) tetravalent target; (b–d) tri-, bi- and monovalent species. Inset: full HPLC run.

2. Reverse thioether ligation in the solid phase

Having proven the usefulness of reverse thioether ligation in solution we have explored its feasibility in the solid phase, which in terms of simplicity and expediency has an edge over solution chemistry. As peptide ligation requires an aqueous medium, the water-compatible ChemMatrix® resin was chosen as the solid support. Functionalisation as a tetrathiol of the Lys dendron core could not be done, as before, by acylation with Trt-Mpa, because the trityl group is not labile enough to 1–2% TFA, and stronger acid concentrations might affect the peptide–resin bond and/or other protecting groups. A further concern was that, at higher TFA concentrations, ethylenglycol-based ChemMatrix® resin could act as a "TFA sponge", also with adverse consequences. The alternative of using unprotected Mpa was problematic as it produced overcoupling to the initial Mpa unit (amide bound to the resin) of (thioester-linked) Mpa units that could not be adequately removed by treatment with either 20% piperidine/DMF or 0.1 M NaOH. A more satisfactory alternative was 3,3′-dithiopropionic acid (DTPA), the solid, odourless dimer of Mpa. Its DIPCI-mediated coupling, followed by reduction with aqueous 0.1 M TCEP, led to the target tetrathiol platform.

With these necessary improvements, the solid phase version of reverse thioether ligation was tested using resin-bound **2** and acetyl-**1** as models (Fig. 5). As above, a 2 eq. per branch excess of ClAc component was used, with four consecutive ligation–reduction cycles, separated by washes. In this scheme, TCEP is never in contact with ClAc groups, hence the aqueous filtrates containing the ClAc peptide can be recycled without any processing. The crude material released from the resin was shown by HPLC (Fig. 5B) to consist almost exclusively of the products of ligation of one-to-four Ac-**1** copies to resin-bound **2**, with the tetravalent target as main product. Thus, while the heterogeneity of the dendrimeric product signals the need for improvement in the number and duration of cycles, and/or the excess of ClAc component, the minimal levels of other impurities must be

viewed as advantageous, even over the solution version. To the best of our knowledge, this is the first account of on-resin ligation of unprotected peptides in aqueous medium, and opens the way to other similar chemoselective ligations.

Experimental procedures

Peptide synthesis and characterization

Automated SPPS was performed in an ABI433 peptide synthesizer (Applied Biosystems, Foster City, CA) running standard Fmoc (FastMoc) protocols[16,27] at 0.1 mmol scale. Side chains of trifunctional residues were protected with trifluoroacetic acid-labile (TFA) *t*-butyl (Asp, Glu, Ser, Thr), trityl (Asn, Gln, His) and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Arg) groups. Lys side chain was protected by either Boc, Fmoc or 4-methoxyltrityl (Mmt) groups, as required. Couplings were done with an 8-fold excess of both Fmoc-amino acid and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and 16-fold excess of *N*,*N*-diisopropylethylamine (DIEA), in *N*,*N*-dimethylformamide (DMF). At the completion of synthesis, peptide resins were N-deblocked with piperidine/DMF (20% v/v) prior to full deprotection and cleavage with TFA/H_2O/TIS (triisopropylsilane) (95 : 2.5 : 2.5 v/v, 90 min, RT). Peptides were precipitated by addition of chilled diethyl ether, taken up in water and lyophilized. Analytical reversed-phase HPLC was performed on C18 or C8 columns (4.6 × 50 mm, 3 μm, Phenomenex, Torrance, CA) in a model LC-2010A system (Shimadzu, Kyoto, Japan). Solvent A was 0.045% (v/v) TFA in water, solvent B was 0.036% (v/v) TFA in acetonitrile. Elution was done with linear gradients of solvent B into A over 15 min (see Table 1 for details) at 1 mL min^{-1} flow rate, with UV detection at 220 nm. Preparative HPLC was performed on Phenomenex C18 (21.2 × 250 mm, 10 μm) or C8 (10 × 250 mm, 10 μm) columns in a Shimadzu LC-8A instrument. Solvents A and B were 0.1% TFA (v/v) in water and acetonitrile, respectively, and elution was again with linear gradients of solvent B into A over 30 min, at 25 or 5 mL min^{-1} flow rate, with UV detection at 220 nm. All peptides were characterised by MALDI-TOF MS in a Voyager DE-STR instrument (Applied Biosystems), using α-hydroxycinnamic acid matrix. Spectra were acquired in the reflector mode for compounds up to MW ~4000 (see Table 1) and in the linear mode for higher MWs.

Chloroacetylated peptides

Peptides **1** and **4** (Table 1) were assembled on Rink amide MBHA resin, with Fmoc-Lys(Mmt) coupled as C-terminal amino by the above Fmoc protocols, followed by the rest of the sequence. At the end of the synthesis, the N-terminal residue was acetylated with acetic anhydride/DIEA (10- and 20-fold molar excess, respectively) and the Mmt group was orthogonally removed with 1% TFA in dichloromethane (DCM). After neutralisation with 5% DIEA in DCM, the Lys ε-amino group was treated with chloroacetic acid and *N*,*N*′-diisopropylcarbodiimide (DIPCI) (10-fold molar excess of both). Final deprotection-cleavage, purification and characterisation were as above.

Thiol-functionalised dendron cores

Lys-branched **2**, **5** and **6** (Table 1) were assembled on Fmoc-Rink-amide ChemMatrix resin by the general procedures above, with branching achieved by Fmoc-Lys(Fmoc) incorporation at the appropriate points. The resulting tetravalent dendron was functionalised as a tetrathiol by treatment with either (i) 3-tritylmercaptopropionic acid/DIPCI; (ii) Mpa/DIPCI followed by 20% piperidine/DMF or 0.1 M NaOH treatment, or (iii) DTPA/DIPCI followed by 0.1 M aqueous solution of TCEP. In all three cases, a 10-fold molar excess of thiol derivative was used. Deprotection–cleavage, purification and characterisation were as above.

Thioether conjugation in solution

For proof-of-principle trials (entries **3**, **7**, **8**, Table 1), 0.5 mg of thiol-derivatised dendron core (0.2–0.8 μmol of thiol functions, depending on size and multiplicity) were dissolved in 10 mL of a 0.02 M solution of $NaHCO_3$, pH 7.5 (readjusted with μL-amounts of 1 M NaOH as required), containing a four- or eight-fold molar excess (2 eq. per branch) of chloroacetylated peptide, and the solution stirred at 50 °C. Reactions were monitored by analytical HPLC and MALDI-TOF MS taking samples of 20 μL of solution and stopping the reaction by addition of the same amount of 10% acetic acid solution. TCEP (0.02 M, 0.5 eq. per branch) was added twice (typically at 2 and 14 h reaction time) to prevent disulfide formation by the dendron. After *ca.* 24 h, conversion was quantitative by analytical HPLC, and the mixture was acidified with acetic acid and lyophilized. The procedure has been scaled up (*ca.* 10×) in subsequent preparative runs, with yields in isolated, HPLC-purified product in the 40–50% range, relative to the thiol-functionalised core.

Thioether conjugation in the solid phase

5 mg of thiol-functionalised dendron core **2** attached to ChemMatrix® resin [prepared by approach (iii) above] were placed in a syringe reactor fitted with a porous polyethylene disk and submitted to four consecutive conjugation–wash–reduction cycles as follows. For each cycle, the conjugation step involved treating the resin with a two-fold molar excess of ClAc-peptide for branch dissolved in the minimal amount required of 0.02 M solution of $NaHCO_3$, pH 7.5, for 1 h at 45 °C, with gentle shaking. The resin was next washed with H_2O (3×), 10% acetic acid (3×), DMF (3×) and H_2O (3×) then treated with 0.02 M solution of TCEP in H_2O for 30 min, then washed with DMF and H_2O before the next cycle. In the third cycle, it was left to react overnight, then treated with TFA/H_2O/TIS (95 : 2.5 : 2.5 v/v, 90 min, RT). The end product (**3**) was precipitated with cold ether, taken up in aqueous AcOH (10% v/v), lyophilised and characterised by HPLC and MS.

Abbreviations

ClAc	chloroacetyl
DIEA	*N*,*N*-diisopropylethylamine
DIPCI	diisopropylcarbodiimide

DTPA	dithiopropionic acid
HBTU	2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate
MALDI-TOF	matrix-assisted laser desorption ionisation time-of-flight
Mmt	4-methoxytrityl
Mpa	mercaptopropionic acid
MS	mass spectrometry
SPPS	solid phase peptide synthesis
TCEP	tris(2-carboxyethyl)phosphine hydrochloride
TFA	trifluoroacetic acid
TIS	triisopropylsilane

References

1 M. H. V. Van Regenmortel and S. Muller, *Synthetic Peptides as Antigens*, Elsevier, Amsterdam, 1999.
2 B. Y. Chua, W. Zeng, Y. F. Lau and D. C. Jackson, *Vaccine*, 2007, **25**, 92.
3 K. Lovgren and M. Larsson, *J. Immunol. Methods*, 1994, **173**, 237.
4 T. Nagata, T. Toyota, H. Ishigaki, T. Ichihashi, K. Kajino, Y. Kashima, Y. Itoh, M. Mori, H. Oda, H. Yamamura, M. Taneichi, T. Uchida and K. Ogasawara, *Vaccine*, 2007, **25**, 4914.
5 F. Borras-Cuesta, Y. Fedon and A. Petit-Camurdan, *Eur. J. Immunol.*, 1988, **18**, 199.
6 J. P. Tam, *Proc. Natl. Acad. Sci. U. S. A.*, 1988, **85**, 5409.
7 J. P. Defoort, B. Nardelli, W. Huang and J. P. Tam, *Int. J. Pept. Protein Res.*, 1992, **40**, 214.
8 K. Rose, W. Zeng, L. E. Brown and D. C. Jackson, *Mol. Immunol.*, 1995, **32**, 1031.
9 J. W. Drijfhout and W. Bloemhoff, *Int. J. Pept. Protein Res.*, 1991, **37**, 27.
10 M. Schnolzer and S. B. Kent, *Science*, 1992, **256**, 221.
11 C. Rao and J. P. Tam, *J. Am. Chem. Soc.*, 1994, **116**, 6975.
12 J. C. Spetzler and J. P. Tam, *Int. J. Pept. Protein Res.*, 1995, **45**, 78.
13 Y. Fujita, P. M. Moyle, S. Hieu, P. Simerska and I. Toth, *J. Pept. Sci.*, 2008, **90**, 624.
14 W. Kowalczyk, M. Monso, B. G. de la Torre and D. Andreu, *J. Pept. Sci.*, 2010, **17**, 247.
15 M. Monso, J. Tarradas, B. G. de la Torre, F. Sobrino, L. Ganges and D. Andreu, *J. Pept. Sci.*, 2011, **17**, 24.
16 W. Kowalczyk, B. G. de la Torre and D. Andreu, *Bioconjugate Chem.*, 2010, **21**, 102.
17 W. Zeng, S. Ghosh, M. Macris, J. Pagnon and D. C. Jackson, *Vaccine*, 2001, **19**, 3843.
18 Y. A. Lu, P. Clavijo, M. Galantino, Z. Y. Shen, W. Liu and J. P. Tam, *Mol. Immunol.*, 1991, **28**, 623.
19 C. Cubillos, B. G. de la Torre, A. Jakab, G. Clementi, E. Borras, J. Barcena, D. Andreu, F. Sobrino and E. Blanco, *J. Virol.*, 2008, **82**, 7223.
20 C. F. Liu and J. P. Tam, *Proc. Natl. Acad. Sci. U. S. A.*, 1994, **91**, 6584.
21 F. Baleux and P. Dubois, *Int. J. Pept. Protein Res.*, 1992, **40**, 7.
22 S. Matysiak, T. Böldicke, W. Tegge and R. Frank, *Tetrahedron Lett.*, 1998, **39**, 1733.
23 S. Matysiak, , T. Böldicke, , W. Tegge and and R. Frank, Innovation and Perspectives in Solid Phase Synthesis & Combinatorial Libraries: Peptides, Proteins and Nucleic Acids–Small Molecule Organic Chemical Diversity, Collected Papers, International Symposium, 5th, London, Sept. 2–6, 1997, 1999, p. 339.
24 E. B. Getz, M. Xiao, T. Chakrabarty, R. Cooke and P. R. Selvin, *Anal. Biochem.*, 1999, **273**, 73.
25 J. A. Burns, J. C. Butler, J. Moran and G. M. Whitesides, *J. Org. Chem.*, 1991, **56**, 2648.
26 D. E. Shafer, J. K. Inman and A. Lees, *Anal. Biochem.*, 2000, **282**, 161.
27 G. B. Fields and R. L. Noble, *Int. J. Pept. Protein Res.*, 1990, **35**, 161.