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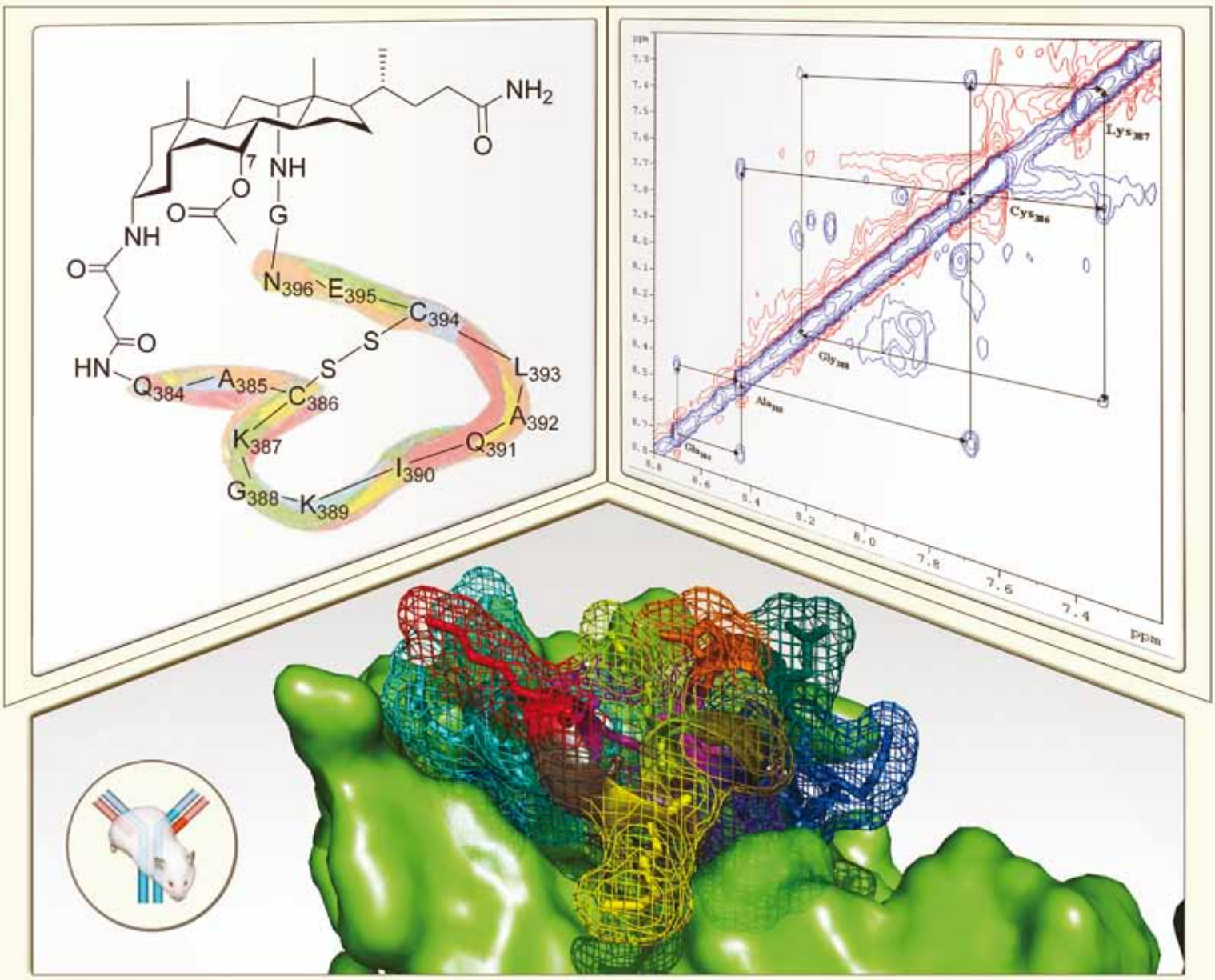

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FULL PAPER

Catherine A. Bodé *et al.*

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Highlights in Chemical Biology

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Towards the conformational mimicry of the measles virus HNE loop: design, synthesis and biological evaluation of a cyclic bile acid–peptide conjugate†

Catherine A. Bodé,[a] Tom Bechet,[b] Emmanuel Prodhomme,[b] Katelijne Gheysen,[c] Pieter Gregoir,[a] José C. Martins,[c] Claude P. Muller[b] and Annemieke Madder*[a]

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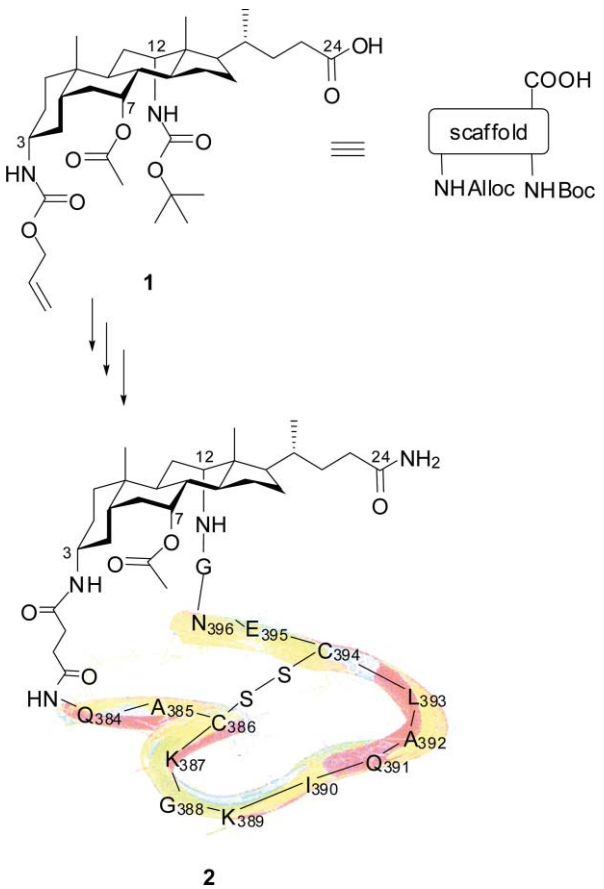
The current article reports the design, synthesis and biochemical evaluation of a cyclic bile acid–peptide conjugate as a mimic of the loop-like structure of the measles virus haemagglutinin noose epitope (HNE). This macrocyclic structure was assembled by solid phase synthesis. Scaffold-peptide ring closure was achieved *via* the introduction of a succinate linker. After disulfide bridge formation with iodine, the desired 14 amino acid cyclic conjugate was obtained with overall yields between 15 and 35%. NMR analysis supports the presence of a helical conformation in the Q384-G388 pentapeptide portion, in agreement with the organisation of this chain in the native protein. The compound was found to have increased biostability compared to stabilised linear peptides, displayed good binding towards monoclonal antibodies known to bind to HNE and thus has potential in an alternative peptide-based measles vaccine.

Introduction

Biologically active peptides are recognised as having significant therapeutic potential.[1] However, the major drawbacks of using naturally occurring peptides *in vivo* include their metabolic instability and their conformational flexibility, alongside their poor bioavailability. The combined effects of these factors entail that peptides have very little chance of producing the desired response when administered on their own. Methods for the improvement of biostability, bioavailability and rigidity of peptides, while preserving peptide activity, are thus highly sought after in immunology and medicinal chemistry.

Cyclic peptides are more stable *in vivo* than their linear counterparts and their reduced conformational mobility allows them to possess functionality in a diverse yet predictable manner.[2] Many efforts in the design of peptide-based drugs concentrate on the optimisation of conformational aspects by involvement of cyclic peptide formation or using templates for conformational restriction.[3] Bioavailability issues such as biostability and membrane permeability are addressed later in the process.

Previous research in our lab focused on the construction of dipodal peptidosteroids as serine protease mimics. This involved attachment of two different peptide chains to orthogonally protected cholic acid derivative **1** (see Scheme 1).[4] In earlier work,



Scheme 1 Conformational restriction of the HNE sequence H384–396 by incorporation onto steroid scaffold **1**. Target molecule **2** is superimposed on the conformation of the H384–396 sequence as determined by molecular modelling (adapted from ref. 12c with permission).

[a]*Laboratory for Organic and Biomimetic Chemistry, Department of Organic Chemistry, Ghent University, B-9000 Gent, Belgium. E-mail: annemieke.madder@ugent.be; Fax: +32 9 264 4998; Tel: +32 9 264 4472*
[b]*Department of Immunology and WHO Collaborative Centre for Measles, Laboratoire National de Santé, L-1950 Luxembourg, Luxembourg*
[c]*NMR and Structure Analysis, Department of Organic Chemistry Ghent University, B-9000 Gent, Belgium*

† Electronic supplementary information (ESI) available: Analytical data for **2**, 2D-NMR study of **2**, synthesis details and analytical data for **18** and details for monoclonal antibody generation and ELISA. See DOI: 10.1039/b907395g

Kramer, Wess, Enhsen and coworkers were pioneers in the design of modified bile acids as peptide carriers. An illustrative example of their investigations into the intestinal absorption of peptides coupled to bile acids is the development of an oxaprolylpeptide–bile acid conjugate.[5] Such conjugates can be regarded as "Trojan horses" for specific drug absorption. Furthermore, it has been shown that linkage to a bile acid protects a peptide backbone against hydrolysis.[6] Based on these findings and on our previous expertise, we here describe the synthesis of cyclic peptides using a steroid moiety as a turn element in an effort to simultaneously address some of the shortcomings of the peptide-based drugs mentioned above. The proposed steroid moiety both serves to conformationally restrict and to increase the bioavailability of the incorporated peptide sequences.[7]

The conformational restriction of the loop-like haemagglutinin noose epitope of the measles virus (MV) haemagglutinin (H) protein, one of the targets of MV-neutralising and protective antibodies, is described here. Although the currently available vaccine has had a dramatic positive impact on protecting countless individuals from this potentially lethal disease, it also bears several shortcomings which are thought to compromise measles eradication.[8] Extensive research has been carried out into alternative vaccine strategies against MV. It has been shown that alternative vaccines can be based on a minimal set of key elements, *i.e.* epitopes represented by synthetic peptides.[9] A measles virus vaccine based on a peptide subunit could possibly circumvent current vaccine drawbacks by being suitable for administration in very young infants even in the presence of transplacentally transmitted maternal antibodies.[10]

Results and discussion

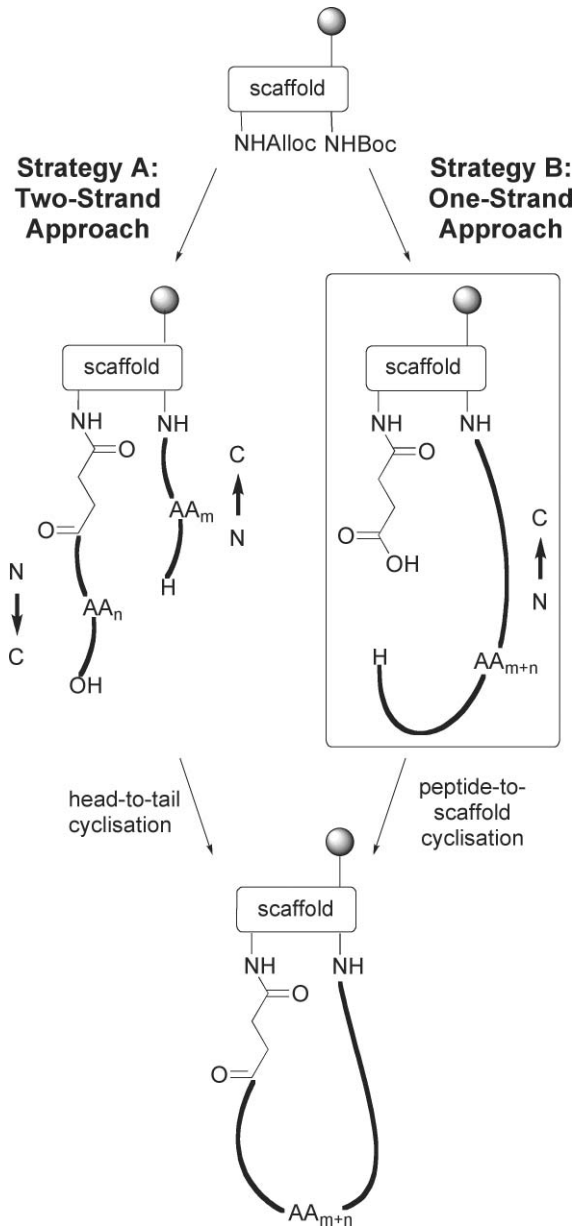
Rationale for the synthesis of a cyclic peptidosteroid as a vaccine candidate against MV

The MV fusion (F) and haemagglutinin (H) membrane proteins mediate virus attachment and penetration. The H protein is highly immunogenic and induces MV-neutralising and protective antibodies.[11] One of the epitopes identified on the H protein is the haemagglutinin noose epitope (HNE), which has been extensively characterised and tested.[12] The peptide sequence was narrowed down to the sequential neutralising and protecting epitope H386–395 (CKGKIQALCE).[12c] Molecular modelling of the core sequence H384–396 revealed the peptide to have an amphiphilic loop-like structure containing a disulfide bridge between cysteines 386 and 394.[12c] Based on these findings, we aim at the development of a new type of peptide-based subunit vaccine **2** by coupling the cystine-bearing HNE core sequence to a non-peptidic entity, the orthogonally protected steroidal scaffold **1** (Scheme 1).

Conformational restriction is achieved through the rigidity of the scaffold, which preorganises the coupled peptide termini in a strictly antiparallel way and allows for the formation of a loop-like structure similar to that of the HNE in its native protein.[12c] The envisaged peptide sequence will be attached at the C-12 position. Introduction of a succinic acid linker allows for cyclisation of the peptide amino terminus onto the second attachment point of the scaffold (C-3). Disulfide bridge formation between the critical cysteine residues can help in preorganising the attached peptide for the final cyclisation onto the scaffold.

Retrosynthesis

Previously, we reported on the synthesis of a related but more simple, cyclic peptidosteroid using a two-strand approach (Scheme 2, strategy A).[13] A similar two-strand approach has recently been reported for the synthesis of a cyclic peptide–furostane conjugate.[14] In the current design the two-strand approach can be considered but a protected peptide methyl ester fragment is needed. In view of the labour intensive synthesis, isolation and purification of the required protected peptide fragment, we here explored the feasibility of the more straightforward one-strand approach (Scheme 2, strategy B).



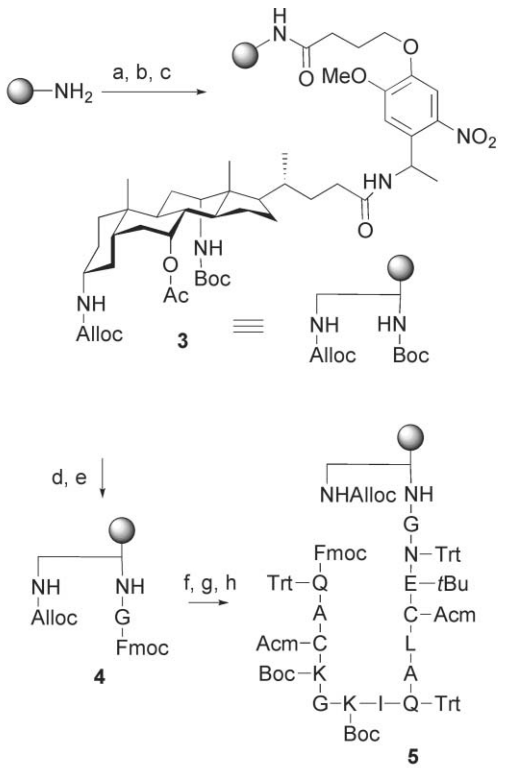
Scheme 2 Construction of the cyclic peptidosteroid either *via* a two-strand approach (strategy A) or a one-strand approach (strategy B).

After attachment of the steroid scaffold **1** to the solid support *via* a suitable linker, construction of the entire peptide onto the amine at the C-12 steroid position will be followed by cyclisation

onto a second amine at C-3. Connection of the two amino groups will occur through a succinate linker.

Synthesis

The selection of a suitable solid support and peptide-resin anchor for a given synthesis is extremely important, quite often governing its outcome. For the purpose of this project, amino-functionalised TentaGel resin was used in combination with a photolabile linker.[15] Introduction of this photocleavable handle ensures complete orthogonality between peptide-resin cleavage and side chain deprotection, allowing convenient cleavage of every synthetic intermediate without affecting side chain protection (Scheme 3). In this way, accurate information on the products actually present on the solid phase can be obtained at every step of the synthesis, including the stability of the side chain protecting groups during the whole synthetic process.



a) 4-{4-[1-(9-fluorenylmethoxycarbonylamino)ethyl]-2-methoxy-5-nitro-phenoxy}butanoic acid, PyBOP, DIPEA, DMF, RT, 2h;
b) 20% piperidine/DMF, RT, 2 x 20 min;
c) methyl-7-α-acetoxy-3α-[N-(allyloxycarbonyl)amino]-12α-[N-(t-butyloxy-carbonyl)amino]-5β-cholan-24-oate, PyBOP, DIPEA, DMF, RT, 2h;
d) 20% TFA, DCM, RT, 20+90 min;
e) Fmoc-Gly-OH, PyBOP, DIPEA, DMF, RT, 3h;
f) 20 % piperidine, DMF, RT, 2 x 20 min;
g) Fmoc-AA-OH, PyBOP or HBTU, DIPEA, DMF, RT, 3h;
h) repeat steps f) and g) until complete.

Scheme 3 Synthesis of the acyclic peptidosteroid precursor.

Coupling of the *o*-nitrobenzyl-based photolinker[15] and subsequent attachment to the scaffold[13] to obtain construct **3** was carried out as previously described. Taking into account both steric considerations and orthogonality between side chain protecting groups in an Fmoc strategy, synthesis started from the C-12 amine. Acid Boc-deprotection was followed by manual coupling of an Fmoc-protected glycine unit. This glycine, which is not part of the critical HNE sequence, functions as a spacer between the scaffold and the attached peptide. Compound **4** was now amenable to automated peptide synthesis. Here, both *N*-[(1H-benzotriazol-1-yl) (dimethylamino) methylene]-*N*-methyl-methanaminium hexafluoro phosphate *N*-oxide (HBTU) and 1H-benzotriazol-1-yloxy-tris(pyrrolidino)phosphonium hexafluoro-phosphate (PyBOP) were employed as coupling reagents. With HBTU occasionally giving rise to guanidinium formation, possibly due to insufficient mixing, PyBOP was the preferred coupling reagent.

For the introduction of the succinate linker, various routes can be envisaged (Scheme 4).

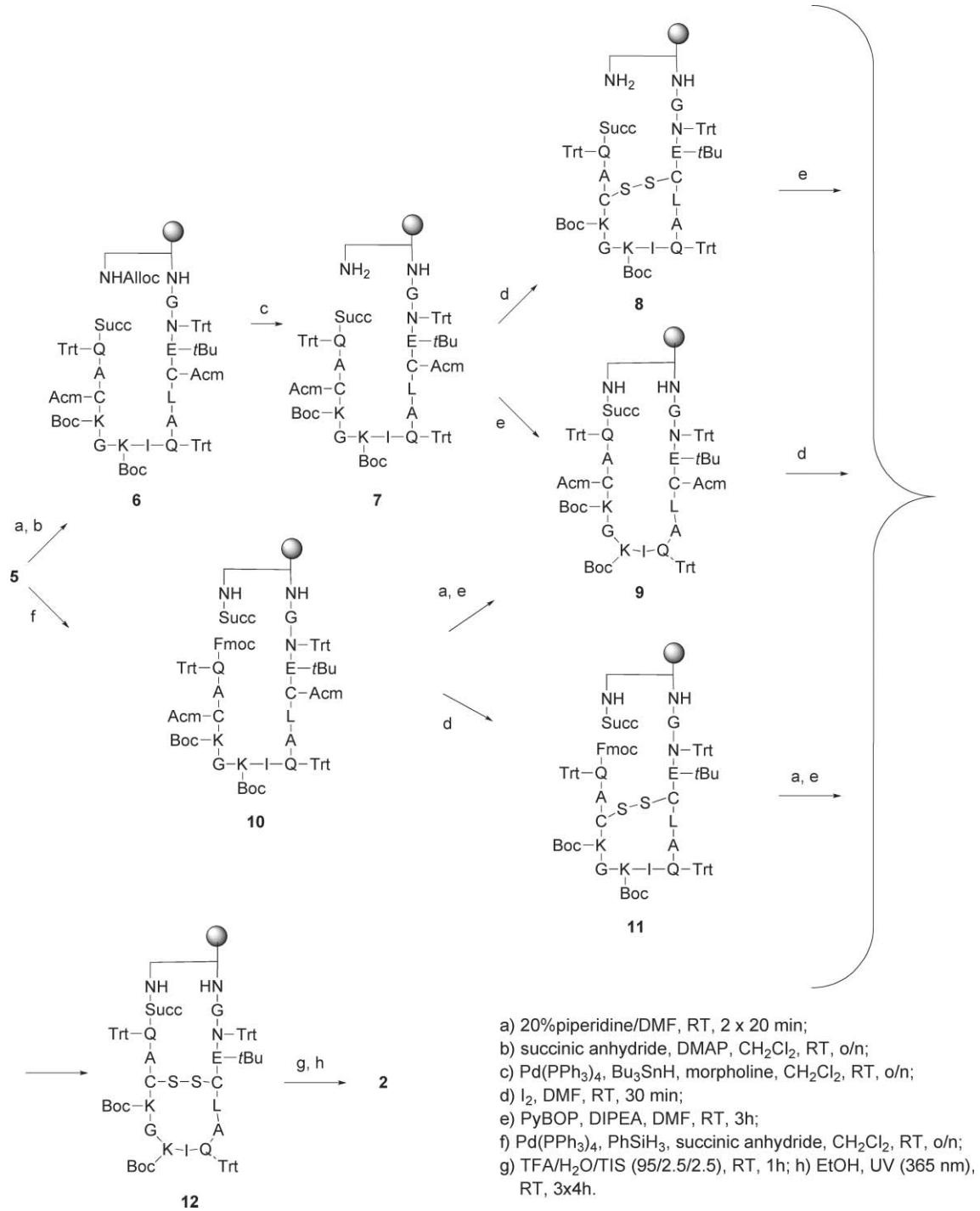
The succinate can be introduced either on the peptide terminus or on the scaffold C-3 amino group. Furthermore, final cyclisation can be performed before or after disulfide bridge formation. The synthetic requirements of the possible routes were investigated and different reaction orders were considered. After Fmoc removal the linker was introduced onto the peptide chain terminus in compound **5** using a large excess of succinic anhydride and 4-dimethylaminopyridine (DMAP) to give complete conversion to compound **6**. Alloc deprotection was effected with tetrakis(triphenylphosphine)-palladium(0) and morpholine. Tributyltin hydride was added to avoid amine allylation and to give complete conversion to **7** in very good purity.

Alternatively the linker can be introduced onto the scaffold C-3 amine. Initial attempts at separate Alloc-removal followed by succinate introduction were found to systematically lead to only partial deprotection, alongside several side reactions. These included partial Fmoc deprotection with succinic anhydride coupling to the terminal peptide amine and allylation of the liberated amines. Therefore, studies were conducted on the simultaneous Alloc removal and linker introduction on **5**. It was found that 2 equivalents of palladium(0) catalyst and phenylsilane as a scavenger gave the desired compound **10** in satisfactory purity.

Disulfide bridge formation can be carried out at various stages along the synthesis. Treatment with iodine, though far less described in conjunction with solid phase-bound peptides under non-acidic conditions, was found to give better purity than thallium(III) trifluoroacetate.[16] Treatment of resins **7** or **10** with 10 equivalents of reagent for 30 min gave complete conversion to **8** and **11** without dimerisation.

The following step of the synthetic sequence was concerned with the linker-mediated peptide-scaffold cyclisation. In the case of compounds **7** and **8**, this takes place between the succinic acid-modified peptide strand and the scaffold C-3 amine. Compounds **10** and **11** in turn are cyclised between the linker-modified C-3 scaffold amine and the peptide glutamine (Scheme 4). Initial test reactions were performed on compound **8** (Table 1).

It was found that treatment with TBTU and diisopropylethylamine (DIPEA) together with 1-hydroxybenzotriazole (HOBt) gave rise to the desired product **12** alongside a substantial amount of an unidentified impurity. HOBt/diisopropylcarbodiimide (DIC) treatment or overnight reaction with PyBOP and DIPEA gave some piperidinyl adduct due to residual piperidine remaining in the sample after Fmoc deprotection. This was avoided when the



Scheme 4 Linker introduction, cyclisation and cleavage of the cyclic peptidosteroid.

reaction time with PyBOP and DIPEA was reduced to 3 h. These reaction conditions were also successfully applied to the cyclisation of compound **11** to give an overall crude product purity of 69%. Under the same conditions, cyclisation of products **7** and **10**, which do not contain a disulfide bridge, also gave the desired product **9** though with more impurities than their cystine-preorganised counterparts (25% overall purity). This reaction pathway therefore also constitutes a viable synthetic strategy for peptide sequences not containing a disulfide bridge.

Deprotection of the acid-labile side chain protecting groups was carried out with a 95% TFA solution with TIS as a scavenger. As premature photolabile linker cleavage was feared, reaction time was reduced to one hour, still giving complete deprotection. The final step of this synthetic pathway was cleavage of the product from the solid phase by irradiation of a resin suspension at 365 nm. DMSO, DMF and ethanol all gave good cleavage results and ethanol was used for large scale cleavages as it dissolved target molecule **2** well.[17] Overall crude yields ranged from 30 to

Table 1 Cyclisation of resin 8 to give compound **12**

	Reagents	Eq.	t	Reaction outcome
1	HOBt	15	o/n	**12** + unidentified impurity
	TBTU	15		
	DIPEA	26		
2	HOBt	6	24 h	**12** + piperidinyl adduct
	DIC	6		
3	PyBOP	6	o/n	**12** + piperidinyl adduct
	DIPEA	12		
4	PyBOP	6	3 h	**12**
	DIPEA	12		

50%. MALDI-TOF analysis indicated the presence of one major compound. Purification by RP-HPLC was performed using a gradient from 20 to 100% B. Product **2** was finally isolated in good purity (see ESI†).

NMR analysis

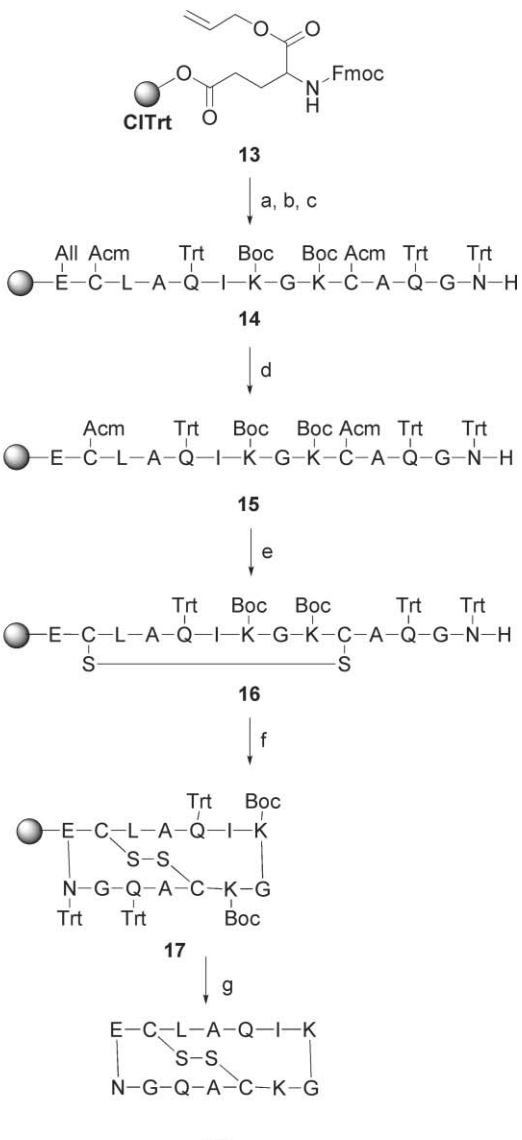
Because of spectral complexity, NMR analysis of **2** focussed on the peptide and its connection to the scaffold, with the entire analysis being limited to the amide region as this is free of contributions from the steroid scaffold. Using the sequence specific resonance assignment approach[18], the Q384-G388 pentapeptide segment was successfully assigned *via* a string of sequential NH_i-NH_{i+1} NOE contacts. The N to C directionality of this sequence was confirmed from αH_i-NH_{i+1} and βH_i-NH_{i+1} NOEs. Unfortunately, from G388 onwards, strong overlap occurs between multiple traces in the TOCSY and NOESY spectra. There is also evidence of line broadening, possibly indicating the presence of conformational exchange linked to the restrictions imposed by its bicyclic nature. As this could not be improved upon by changing temperature, the assignment was limited to the pentapeptide only.

Having been synthesised *via* the one strand approach, closure of the peptide loop onto position 3 of the steroid scaffold could also be established unambiguously. Indeed, two clear NOE contacts linking the amide of Q384 and two resonances at 2.53 and 2.38 ppm can be identified. The latter are not connected to an amide in the COSY or TOCSY spectrum, proving they are not part of an amino acid side chain. In addition, they each show an NOE to the amide at position 3 (8.10 ppm) of the steroidal scaffold (see ESI† for an illustrative combined 2D TOCSY/2D NOESY spectrum). Together with additional NOE contacts between the methylenes in the succinic acid moiety and the Q384 side chain, and the fact that the mass found is that expected from **2**, this data conclusively establishes correct ring closure and integrity of the peptide chain. Finally, it should be noted that the string of sequential NH_i-NH_{i+1} NOE contacts supports the presence of a helical conformation in the Q384-G388 pentapeptide, in agreement with the organisation of this chain in the native protein (see ESI† for an illustrative 2D NOESY spectrum).

Biochemical evaluation

Synthesis of cyclic control sequence 18. The synthesis of a purely peptidic counterpart of compound **2** was undertaken, containing the cyclised HNE sequence without the steroidal scaffold. This structure was used to evaluate the influence of the scaffold on antibody binding. Also, the biostability of the two molecules can be compared to define the impact of the steroidal template on metabolic stability.

The synthetic pathway for backbone-cyclised compound **18** consisted of the coupling of Fmoc-Glu_{395}-OAll *via* its side chain to chlorotrityl resin followed by classical chain elongation (Scheme 5) to give compound **14**. This permitted selective deprotection of the allyl group with Pd(0). Cystine formation was then carried out to give compound **16** without dimerisation. At this stage backbone cyclisation was carried out to give molecule **17** and the control peptide **18** was obtained by side chain deprotection and resin cleavage with TFA.



a) 20 % piperidine, DMF;
b) Fmoc-AA(prot)-OH, PyBOP, DIPEA, DMF;
c) repeat steps a) and b) until sequence complete;
d) $Pd(PPh_3)_4$, $PhSiH_3$, CH_2Cl_2;
e) I_2, DMF; f) PyBOP, DIPEA, DMF; g) TFA/H_2O/TIS.

Scheme 5 Synthesis of a cyclic control sequence without steroid scaffold.

With both target molecule **2** and its purely peptidic counterpart **18** in hand, the first tests were carried out in order to assess the potential of our candidate and to compare it to previously tested linear counterparts.

Biostability tests. The metabolic stability of a compound is a major determinant of its biological activity. In this context, the metabolic stability of compound **2** is of great interest, as the influence of peptide cyclisation and inclusion of a non-peptidic entity can be evaluated. In order to estimate and compare the digestive resistance of the conjugate molecule **2** and of its cyclised analogue without scaffold **18** in an *in vivo* environment, both compounds were incubated with mouse serum at 37 °C and analysed by HPLC at the outset and after 24 h. The stability was then calculated by integration of the HPLC peaks obtained from equal injection volumes. Results indicate that over 75% of both cyclic peptides **2** and **18** resist degradation in the serum for 24 h. This is a significant improvement over the natural peptide, which was degraded to 25% within the same time of incubation. For comparison, linear stabilised peptides degrade between 20 and 80% within the first 24 h (data not shown).[19]

Monoclonal antibody binding. A classical indirect ELISA protocol was used to assess binding of antibodies BH216, BH6 and BH21 to the peptide constructs **2** and **18**. These monoclonal antibodies (mAb) bind with different fine-specificities to the HNE epitope of the intact haemagglutinin protein of MV. The three antibodies neutralise the virus and protect mice against a lethal challenge with a rodent adapted virus. They also bind to peptides corresponding to the HNE epitope and cyclised *via* their cysteine residues. Such a linear HNE peptide analogue was used as a positive control. mAb BH195, known to bind only to non-cyclised HNE peptide and selectively to the H397–400 region of the denatured H-protein, was used as a negative control antibody.

From Fig. 1A and Fig. 1B it can be deduced that the binding curves of BH216 and BH21 are comparable, with the target molecule **2** giving the strongest binding, followed by the linear control peptide. Cyclic peptide **18** gave a much weaker binding. Binding to BH6 was maximal and almost equivalent for both **2**, its cyclic counterpart without scaffold **18**, and the wild-type control peptide (Fig. 1C). The control mAb BH195, showed essentially no reactivity, confirming the specificity of the reactivity of the mAbs (Fig. 1D). The biochemical behaviour of peptide conjugate **2** thus makes it a strong candidate for further immunological evaluation *in vivo* in conjunction with a T-cell activator.

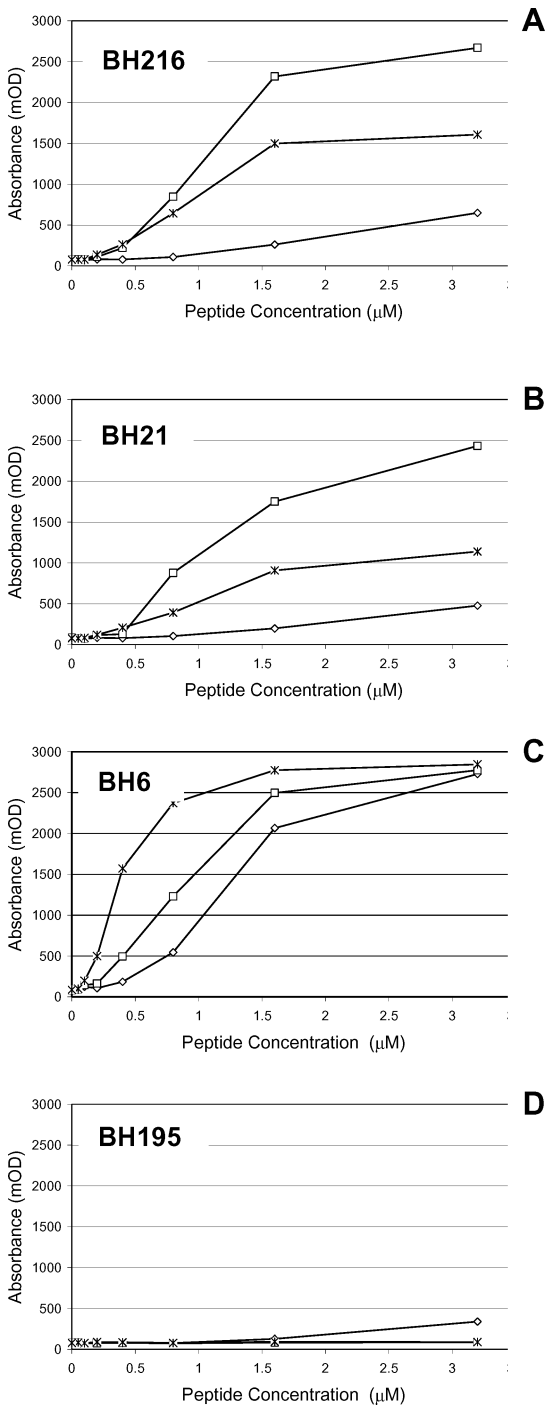


Fig. 1 Binding of monoclonal antibodies BH216 (**A**), BH21 (**B**), BH6 (**C**) and BH195 (**D**) to compounds **2** (□) and **18** (◇) and to linear control peptide (★).

Conclusions

The cyclic peptidosteroid conjugate **2** was successfully synthesised by solid phase synthesis. 2D-NMR analysis not only allowed us to confirm correct cyclisation to the peptide steroid scaffold but additionally supports the presence of a helical conformation in the Q384-G388 pentapeptide portion, in agreement with its occurrence in the native protein. The conjugate was further shown to present good biostability and improved binding to monoclonal antibodies known to bind to the neutralising and protective sequential MV HNE epitope. The developed synthetic route represents a general strategy towards the generation of stabilised loop-like peptide structures for the conformational mimicry of peptides, allowing for flexibility in the choice of peptide sequence, linker and scaffold. These stabilised mimics thus have great potential for the generation of antigenic and immunogenic peptides. In order to test for immunogenicity, conjugation with a T-cell epitope is required. The current encouraging results form the basis of the design of a more elaborate analogue featuring an extra handle at the cholic acid side chain that allows for further convergent coupling to a

suitable peptide sequence, results upon which we will report in the near future.

Experimental section

Materials and methods

Chemicals and reagents. TentaGel S NH_2 (capacity ~0.25 mmol/g, 90 μm) and Polystyrene PHB (Wang resin, capacity ~0.99 mmol/g, mesh 100–200) were obtained from RAPP Polymere GmbH or Iris Biotech GmbH. All amino acids were purchased from Novabiochem. L-amino acids were used throughout the synthesis. DMF, extra dry with molecular sieves, was obtained from Acros. Dichloromethane was distilled from CaH_2. All chemicals were used without further purification, unless otherwise stated. All reagents used for automated peptide synthesis were peptide synthesis grade.

Solid phase peptide synthesis. For manual peptide synthesis, reactions were performed in a peptide vessel protected against light and comprising a sintered glass funnel and a 3-way stopcock for easy filtration and washing or in plastic vials equipped with a sintered filter. The solid phase reactions were performed on a shaker (Selecta Vibromatic) at 200 U/min or on a Yellow Line TTS 2 vortexer at 1200 rpm. Automated peptide syntheses were performed on a 96-reactor block on a SYROII robot or on a 48-reactor block SYRO Multiple Peptide Synthesiser equipped with a vortexing unit (both Multisyntech, Witten, Germany). Colour tests were used to allow fast assessment of reaction progress. Both the TNBS test and the NF31 test were applied.

Peptide characterisation. Photolyses were carried out on a small scale with a 4 W Bioblock Scientific compact UV lamp set at 365 nm. For large scale cleavage, a 450 W UV ACE glass incorporated 7225–34 immersion lamp equipped with a Schott WG320 UV cut-off filter was used. All samples were cleaved at a distance of 1 cm from the lamp. Resin loadings were determined with a Varian Cary 3E UV/VIS spectrophotometer. ESI-MS spectra were recorded using an LCQ ion trap mass spectrometer (Finnigan MAT). MALDI-TOF spectra were recorded on an Applied Biosystems Voyager-DE STR Biospectrometry Workstation, using 2,5-dihydroxybenzoic acid as matrix. For peptidic compounds, the exact mass, rather than the molecular weight of the compound is stated, as this allows for easier evaluation of mass spectrometric data. When working with the photolabile linker, the exact mass of the amide resulting from UV cleavage is given. All NMR analysis was performed at 500 MHz on a Bruker DRX 500 equipped with a 5 mm ^{1}H, ^{13}C, ^{15}N TXI-Z gradient probehead. The sample was prepared from 1.5 mg of **2** obtained *via* the one strand approach and was dissolved in 600 μL of DMSO-d_6, affording a 1.25 mM solution. Spectra were recorded at 298K, with DSS as internal standard and consisted of 1D ^{1}H, 2D gCOSY, a 2D TOCSY with 100 ms MLEV-17 spin lock and a 300 ms 2D NOESY spectrum were recorded using established procedures.[20] All data were acquired and processed using TopSpin 1.3.

Unless otherwise stated, RP-HPLC analyses were performed on an Agilent 1100 Series instrument with a Phenomenex Luna C18(2) column (250 × 4.6 mm, 5 μm at 35 °C). A flow rate of 1 mL/min and with the following solvent systems: 0.1% TFA in H_2O (A) and MeCN (B). The column was flushed for 3 min with 100% A, then a gradient from 0 to 100% B over 15 min was used, followed by 5 min of flushing with 100% B. The identity of peaks was confirmed by analysing collected fractions by MALDI-TOF. Alternatively, a Phenomenex Jupiter C4 column (250 × 4.6 mm, 5 μm at 35 °C) was used. Semi-preparative purification was carried out on two consecutive Phenomenex Bondclone 10 C18 columns (300 × 7.8 mm at 35 °C) using a flow rate of 2.9 mL/min.

LC-MS data were collected on an Agilent 1100 Series instrument with a Phenomenex Luna C18(2) column (250 × 4.6 mm, 5 μm at 35 °C) and an ES-MSD type VL mass detector using the following solvent systems: 5 mM NH_4OAc in H_2O (A) and MeCN (B). The column was flushed with 100% A for 2 min, then a gradient from 0 to 100% B over 15 min was used, followed by 5 min of flushing with 100% B.

Peptide synthesis

TentaGel-photolinker-scaffold (3). To TentaGel-NH_2 (3.27 g, 0.26 mmol/g) were added DMF (30 mL), the photolabile linker 4-{4-[1-(9-fluorenylmethoxy-carbonylamino)ethyl]-2-methoxy-5-nitrophenoxy}-butanoic acid (V.6) (1.32 g, 2.55 mmol), PyBOP (1.32 g, 2.55 mmol) and DIPEA (0.84 mL, 5.09 mmol). The reaction mixture was shaken at r.t. for 2 h, after which the resin was washed. Washing times were extended to 3 × 5 min. The TNBS and NF31 tests remained colourless. The loading was determined by UV absorbance at 300 nm and was found to be 0.21 mmol/g, corresponding to a coupling yield of 91%.

The resin (2.50 g, 0.21 mmol/g) was treated with a solution of 20% piperidine in DMF (30 mL) for 2 × 20 min. The resin was washed. TNBS test gave dark orange beads.

To the deprotected resin was added DMF (16 mL), methyl 7-α-acetoxy-3α-[*N*-(allyloxycarbonyl)amino]-12α-[*N*-(t-butyloxycarbonyl)amino]-5β-cholan-24-oate (1.01 g, 1.60 mmol), PyBOP (0.83 g, 1.60 mmol) and DIPEA (0.27 mL, 3.20 mmol). The reaction mixture was shaken at r.t. for 2 h, after which the resin was washed. Washing times were extended to 3 × 5 min. The TNBS and NF31 tests remained colourless.

Coupling of Fmoc-Gly-OH (4). To a suspension of resin **3** (2.565 g, 0.19 mmol/g) in DMF (2.5 mL) were added Fmoc-Gly-OH (0.432 g, 1.454 mmol), PyBOP (0.756 g, 1.454 mmol) and DIPEA (0.480 mL, 1.454 mmol). The mixture was left to react for 2 h and was then washed. The NF31 test remained colourless. A small sample was UV-irradiated for analysis: t_R: 20.4 min; ESI-MS: calcd for $C_{47}H_{62}N_4O_8$: 810.46, found: ESI: *m/z* 810.9 $[M + H]^+$, 834.4 $[M + Na]^+$; MALDI-TOF: 812.7 $[M + H]^+$, 834.7 $[M + Na]^+$, 850.7 $[M + K]^+$.

Peptide chain elongation (5). Resin **4** (200 mg, 0.18 mmol/g) was transferred to an automated peptide synthesiser for chain elongation. For Fmoc deprotection, the reactor was filled with a 0.8-fold coupling volume of a solution of 40% piperidine in DMF and left to react for 3 min. The solution was removed by filtration under reduced pressure and deprotection was repeated with a 0.8-fold coupling volume of a solution of 20% piperidine in DMF and left to react for 12 min, after which the solution was removed by filtration under reduced pressure and the resin was washed 6 times with DMF (1.1-fold coupling volume) for 30 s each. For

amino acid coupling, the resin was treated with a solution of Fmoc-amino acid in DMF (0.5M), PyBOP in DMF (0.5M) and DIPEA in NMP (2M). 5 equivalents of amino acid and coupling reagent were used and 10 equivalents of base. The resin was left to react for 40 min. The reaction mixture was removed under reduced pressure and the resin was washed 4 times with DMF (1.1-fold coupling volume) for 30 s each. The TNBS and NF31 tests remained colourless. A small sample was UV-irradiated for analysis: t_R: 23.2 min (Jupiter column); ESI-MS: calcd for $C_{181}H_{236}N_{24}O_{32}S_2$: 3321.70, found: MALDI-TOF: 3347.6 $[M + Na]^+$.

Linker coupling to peptide terminus (6). To resin **5** (146 mg, 0.12 mmol/g) suspended in CH_2Cl_2 (3 mL) were added succinic anhydride (170 mg, 1.7 mmol) and DMAP (249 mg, 2.04 mmol). The reaction mixture was shaken overnight after which the excess reaction mixture was filtered off under reduced pressure and the resin was washed. A small sample was UV-irradiated for analysis: t_R: 21.8 min; ESI-MS: calcd for $C_{170}H_{230}N_{24}O_{33}S_2$: 3200.66, found: MALDI-TOF: 3224.9 $[M + Na]^+$, 3240.9 $[M + K]^+$.

Disulfide bridge formation (8 and 11). Resins **7** and **10** (200 mg, 0.11 mmol/g each) were first terminally deprotected. Therefore the resin was treated with a solution of 20% piperidine/DMF (2 mL) for 20 min. The solution was removed by filtration and the resin was washed once with DMF (4 mL). The resin was treated with a solution of 20% piperidine/DMF (2 mL) for 20 min. The solution was removed by filtration under reduced pressure and the resin was washed six times with DMF (4 mL). The resin, previously swollen in DMF, was then treated with a solution of I_2 (56 mg, 0.22 mmol) in DMF (2 mL). The reaction mixture was shaken for 30 min. After reaction, all excess reagents and solvents were removed by filtration under reduced pressure and the resin was washed with DMF (3 × 30 s, 4 mL), MeOH (3 × 30 s, 4 mL) and DCM (3 × 30 s, 4 mL).

A small sample of **8** was UV-irradiated for analysis: t_R: 17.0 min; MALDI-TOF: calcd for $C_{160}H_{214}N_{22}O_{29}S_2$: 2971.54, found: 2974.1 $[M + H]^+$, 2997.0 $[M + Na]^+$.

Scaffold-peptide cyclisation (12). To a suspension of resin **8** or **11** (135 mg, 0.13 mmol/g and 280 mg, 0.10 mmol/g, respectively) in DMF (2 mL) were added PyBOP (53 mg, 0.102 mmol) and DIPEA (36 µL, 0.204 mmol). The reaction mixture was shaken for 3 h and the excess reaction mixture was removed by filtration under reduced pressure, followed by washing the resin. A small sample was UV-irradiated for analysis: t_R: 27.9 min; MALDI-TOF: calcd for $C_{160}H_{212}N_{22}O_{28}S_2$: 2953.53, found: 2977.0 $[M + Na]^+$, 2992.7 $[M + K]^+$.

Linker coupling to scaffold (transacylation, 10). Succinic anhydride (220 mg, 2.2 mmol), phenylsilane (68 µl, 0.55 mmol) and $Pd(PPh_3)_4$ (51 mg, 44 µmol) were added in this order to resin **5** (200 mg, 0.11 mmol/g) in CH_2Cl_2 (3 mL) under inert atmosphere. The reactor was immediately flushed with argon and left to shake overnight. The resin was then filtered and washed until the resin turned pale yellow. A small sample was UV-irradiated for analysis: t_R: 31.1 (Luna, broad), 22.5 min (Jupiter); MALDI-TOF: calcd for $C_{181}H_{236}N_{24}O_{33}S_2$: 3337.70, found: 3362.7 $[M + Na]^+$, 3378.6 $[M + K]^+$.

Side chain deprotection, cleavage and purification (2). Resin **12** (250 mg, 0.15 mmol/g) was treated with a solution of TFA/H_2O/TIS (95/2.5/2.5) (3 mL) at r.t. for 1 h, after which the resin was washed. A small sample was UV-irradiated for analysis: t_R: 14.5 min; MALDI-TOF: calcd for $C_{89}H_{146}N_{22}O_{24}S_2$: 1971.03, found: 1994.9 $[M + Na]^+$, 2009.9 $[M + K]^+$.

Resin **12** (200 mg, 0.17 mmol/g) was suspended in EtOH (6 mL). Nitrogen was gently bubbled through the reaction vessel to keep the resin suspended. Fresh EtOH (4 mL) was added whenever it had evaporated. The reaction vessel was irradiated with UV light at 365 nm for 4 h. The filtrate was collected and the resin was washed with EtOH (4 × 3 mL). The combined filtrates were evaporated under reduced pressure to give 7.5 mg of crude product **2**. The cleavage was repeated twice to give 3.6 and 0.9 mg of product **2**, giving a cleavage yield of 33%.

For the purification, 7 mg of crude product **2** were dissolved in 0.5 ml water and was purified by RP-HPLC in 5 injections of 100 µL each with a gradient of 20 to 100% B over 15 min. Fractions were collected between 10 and 11 min and all solvents were lyophilised to give 4.4 mg pure **2** in a purification yield of 63% and in an overall yield of 21%. The compound was further characterised using NMR spectroscopy (see ESI†).

Peptide stability

30 µL of a 1mg/mL solution of sample in water was mixed with 30 µL of mouse serum in a 200 µL tube. The mixture was left to incubate at 37 °C. 15 µL samples were collected after 0 and 24 h. Each sample was diluted with 335 µL HPLC solvent A and filtered through a 30 kDa size-exclusion filter by centrifuging at 13 000 rpm for 10 min at 20 °C. 300 µL of filtrate containing approximately 6.5 µg of peptide was used for analysis by RP-HPLC on an ÄKTAexplorer 10S system (Amersham Biosciences, Uppsala, Sweden), equipped with an F950 fraction collector (Amersham Biosciences, Uppsala, Sweden). The following solvent systems were used: solvent A: water, 0.1% TFA and solvent B: water, 80% MeCN, 0.1% TFA. The analytical column used was a Gromsil 120 ODS-4 HE (C18) (250 × 4 mm, 5 µm). Biostability tests were performed on a Zorbax 300SB-C3, (150 × 2.1 mm, 5 µm) and the analytical column mentioned above. Biostability was expressed as percentage of remaining intact peptide determined *via* automated integration of the corresponding HPLC peak.

ELISA. ELISA was performed using a previously described method.[12c] In brief, 96-well plates (Maxisorp, Nalge Nunc, Rochester, NY, USA) were used. Carbonate-bicarbonate buffer (CB) was adjusted to pH 9.6 and washing buffer (WB) contained 154 mM NaCl, 1 mM TrisBase (2-amino-2-(hydroxymethyl)propane-1,3-diol), 1.0% Tween-20 (polyoxyethylene(20) sorbitan monolaurate) at pH 8.0. Blocking buffer (BB) was a solution of 136 mM NaCl, 2 mM KCl, 15 mM Tris-Acetate (tris(hydroxymethyl)aminomethane acetate), 1.0% BSA, adjusted to pH 7.4. Dilution buffer (DB) was a solution of BB and 0.1% Tween-20. mAb as primary antibody was used in a 1 : 1000 dilution in DB. As secondary antibody, goat anti-mouse IgG-AP was used (1 : 750 dilution; Southern Biotechnology, Birmingham, AL, USA) in DB. A 1.35 mM phosphatase substrate (Sigma-Aldrich) in substrate buffer (SB), containing 1 mM AMP (2-amino-2-methyl-1-propanol), 0.1 mM $MgCl_2 \cdot 6H_2O$ adjusted to pH 10.2, was used. Optical density was measured at 405 nm on

a SPECTRAmax PLUS[384] microplate reader system (Molecular Devices, Sunnyvale, CA, USA).

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