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Validation of a solid-phase-bound steroid scaffold for the synthesis of novel cyclic peptidosteroids‡

CATHERINE A. BODÉ,[a] CLAUDE P. MULLER[b] and ANNEMIEKE MADDER[a]*

[a] Laboratory for Organic and Biomimetic Chemistry, Department of Organic Chemistry, Ghent University, B-9000 Gent, Belgium
[b] Institute of Immunology and WHO Collaborative Center for Measles, Laboratoire National de Santé, L-1950 Luxembourg, Luxembourg

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Abstract: The current article reports on the synthesis of a new type of cyclic peptidosteroid, in which a bile-acid-based scaffold was used for the conformational restriction of a loop-like peptide. Convergent coupling of two tetrapeptides to the non-peptidic steroidal entity was carried out once in the classical C-to-N and once in the non-classical N-to-C direction. Peptide backbone cyclisation was then carried out, giving rise to a ring size equivalent to approximately 12 amino acids. This type of construct will be used in the development of a peptide vaccine against measles. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: convergent coupling; backbone cyclisation; solid-phase peptide synthesis; bile-acid scaffold; peptide delivery

INTRODUCTION

In spite of widespread vaccination with the live attenuated measles vaccine and a dramatic decrease in measles prevalence and mortality, the MV continues to kill 475 000 persons a year. In order to achieve the WHO target of complete measles elimination, there is a common consensus that alternative vaccination strategies have to be explored to counter current vaccine drawbacks [1]. The use of BCEs in a peptide-based vaccine could potentially constitute a useful alternative to current vaccine strategies [2]. In this context, HNE, a sequential BCE spanning residues H381–400 and forming a loop-like structure held together by a disulfide bridge [3] has been found to induce neutralising antibodies against MV wild-type isolates [4].

However, the applicability of linear peptides as drugs is potentially limited by their poor oral bioavailability, and susceptibility to proteolytic cleavage. Moreover, removed from the structural context within its native protein, a peptide can adopt multiple conformations of which only a few still possess the desired bioactive conformation. Considerable efforts have therefore been made in the field of peptide–carrier conjugation in order to prevent peptide degradation, improve the peptides' membrane-passing properties and conformationally restrict them [5].

Bile acids are known to be absorbed in the small intestine by a secondary active Na^+-dependent uptake mechanism. By covalently coupling them to peptides, they could therefore be used as a shuttle system to increase intestinal peptide absorption. Moreover, conjugation of a peptide with a bile acid could prevent the former's pre-systemic degradation [6]. And finally, there is evidence from crystal structure analysis that the rigidity and configuration of bile-acid structures make them ideal candidates for the conformational restriction of peptides attached to them [7]. It is therefore not surprising that steroids are considered to be ideal synthons for diverse conjugates with pharmacological applications [8]. Cholic acid has earlier been validated for the convergent coupling of linear epitope sequences [9]. As for cyclic peptidosteroids, Wess reported on the synthesis of cyclic peptidosteroids comprising two amino acids between C-3 and C-7 [10]. Furthermore, the synthesis of a macrocyclic steroidal derivative involving the two secondary hydroxyl groups in deoxycholic acid (C-3 and C-12) has been reported, though in low yield [11].

The aim of the present research is to synthesise a new type of cyclic peptidosteroids, such as model compound **12** encompassing scaffold **1** (Figure 1) and a loop-like peptide sequence connecting positions C-3 and C-12. The current synthetic route serves as a preliminary study towards the convergent coupling of longer peptide sequences to steroidal structures and their subsequent intra-strand backbone cyclisation. This will eventually lead to the development of a novel peptide-based vaccine against MV, comprising the

Abbreviations: BCE, B-cell epitope; HNE, haemagglutinin noose epitope; MV, measles virus; TIS, triisopropylsilane; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

* Correspondence to: A. Madder, Laboratory for Organic and Biomimetic Chemistry, Department of Organic Chemistry, Ghent University, Krijgslaan 281 (S4), B-9000 Gent, Belgium;
e-mail: annemieke.madder@ugent.be

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aforementioned HNE in its native loop-like structure. In the long term, the methodology will be applicable for the conformational pre-organisation of other peptide sequences.

MATERIALS AND METHODS

TentaGel S NH_2 (capacity: 0.25 mmol/g) 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. DMF extra dry with molecular sieves was obtained from Acros. All chemicals were used without further purification, except for dichloromethane, which was distilled from CaH_2.

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: A solution of 20% piperidine/DMF was added to a sample of resin to give a concentration of 1.5 mM. The mixture was swirled occasionally. After 30 min, the resin was left to settle and the solution was transferred to a UV cuvette. The absorption of the piperidine–dibenzofulvene adduct was measured at 300 nm and the resin loading was calculated from a previously established calibration curve. Reactions carried out in solution were followed by thin layer chromatography on glass plates pre-coated with silica gel (60F254, 0.25 mm). 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. RP-HPLC analyses were performed on an Agilent 1100 Series instrument with a Phenomenex Luna C18(2) column (250 × 4.6 mm, 5 μ at 35 °C) using 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 the collected fractions by MALDI-TOF. LC-MS data were collected on an Agilent 1100 Series instrument with a Phenomenex Luna C18(2) column (250 × 4.6 mm, 5 μ 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, followed by 5 min of flushing with 100% B.

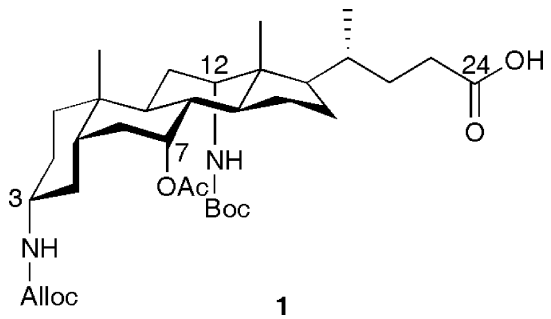

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Figure 1 Bile-acid-based scaffold.

For reaction monitoring, two different colour tests were used. For the TNBS test, approximately 1 mg of resin beads was transferred to a small glass test tube. This was treated with 10 μl of 10% DIPEA/DMF and 10 μl of 1% TNBS/DMF at room temperature (r.t.). After 5 min, the excess solvent was removed and the resin was repeatedly washed with small amounts of DMF and MeOH. The amount of free amine was estimated by comparison of the resin colour with that of reference resins [12,13]. For the NF31 test, approximately 1 mg of resin beads was transferred to a small glass test tube. To this were added 100 μl of a 0.002 M solution of NF31 (4-nitrophenyl-5-{*N*-ethyl-*N*-[4-(4-nitrophenyl)azo]phenyl}amino-3-oxapentoate) in MeCN. The mixture was heated for 10 min at 70 °C. The excess solvent was removed and the resin was repeatedly washed with small amounts of DMF and MeOH. The amount of free amine was estimated by comparison of the resin colour with that of reference resins [14].

All resins were swollen prior to reaction for 30 min in the solvent to be used for the reaction. Reactions were performed in a peptide vessel protected against light and comprising a sintered glass funnel and a three-way stopcock for easy filtration and washing. Unless otherwise stated, after reaction all excess reagents and solvents were filtered off and the resin was washed with DMF (3 × 30 s), MeOH (3 × 30 s) and DCM (3 × 30 s). 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.

Resin-Linker (2)

To TentaGel-NH_2 (3.27 g, 0.26 mmol/g) was added DMF (30 ml), the photo-labile linker 4-{4-[1-(9-fluorenylmethoxycarbonylamino)ethyl]-2-methoxy-5-nitrophenoxy}-butanoic acid (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 loading was determined by UV absorbance and was found to be 0.21 mmol/g, corresponding to a coupling yield of 91%. TNBS test: colourless; NF31 test: colourless.

Resin-Linker-Scaffold (3)

Resin **2** (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 and gave dark orange beads upon treatment with TNBS. 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** (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. TNBS test: colourless; NF31 test: colourless.

Fmoc-Gly_4-OH (4)

Tetraglycine (122 mg, 0.50 mmol) and Na_2CO_3 (105 mg, 0.99 mmol) were dissolved in water (5 ml). To this solution was added a solution of Fmoc-*O*-succinimide (184 mg, 0.55 mmol)

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in dioxane (5 ml). The reaction mixture was stirred at r.t. for 21 h. The dioxane was evaporated under reduced pressure. The remaining mixture was acidified to pH 2 with citric acid, upon which the solute precipitated as a white solid. The solid was filtered off and washed repeatedly with 1 M HCl. The obtained filtrate was evaporated under reduced pressure with toluene to remove any residual water to give a white solid (201 mg, 0.43 mmol) in a crude yield of 86% and could be used without further purification. Exact Mass: 468.2; ESI-MS: 491.1 (100) $[M + Na]^+$, 513.1 (45) $[M - H + 2Na]^+$, 528.9 (15) $[M - H + Na + K]^+$, 958.5 (40) $[MM + Na]^+$, 1003.1 (40) $[MM - 2H + 3Na]^+$, 1448.3 (15) $[MMM - H + 2Na]^+$, 1494.0 (10) $[MMM - 3H + 4Na]^+$; TLC (MeOH): $R_f = 0.63$; RP-HPLC: $R_t = 15.4$ min; LC-MS: $R_t = 11.8$ min.

Boc Deprotection and Coupling of Fmoc-Gly$_4$-OH (5)

Resin **3** (2.50 g, 0.18 mmol/g) was treated twice with a solution of 20% TFA in DCM (30 ml) for 20 and 90 min, respectively. The resin was washed with DCM (3 × 5 min), 10% DIPEA/DMF (3 × 5 min), DMF (3 × 5 min), MeOH (3 × 5 min) and DCM (3 × 5 min). The NF31 test gave dark red beads. Fmoc-Gly$_4$-OH (**4**) (16 mg, 35 μmol) was pre-activated in a round-bottomed flask with HOAt (5 mg, 35 μmol) and DIC (5 μl, 35 μmol) in DMF (3 ml). The reaction mixture was stirred at 0 °C for 15 min and was left to come to r.t. while stirring for another 15 min. The mixture was added to the Boc-deprotected resin (275 mg, 0.18 mmol/g) and was shaken at r.t. overnight, after which the resin was washed. Exact Mass: 981.5; NF31 test: colourless; ESI-MS: 982.1 (15) $[M + H]^+$, 1004.3 (100) $[M + Na]^+$; RP-HPLC: $R_t = 19.1$ min; LC-MS: $R_t = 16.6$ min.

Alloc Deprotection and Succinate Coupling (6)

To the pre-swollen resin **5** (275 mg, 0.17 mmol/g) were added DCM (2 ml), succinic anhydride (50 mg, 0.50 mmol), Bu_3SnH (133 μl, 0.50 mmol) and $Pd(PPh_3)_4$ (11 mg, 10 μmol). The reaction mixture was flushed with argon and left to shake at r.t. for 17 h, after which it was washed. Exact Mass: 997.52; NF31 test: colourless; TNBS test: colourless; ESI-MS: 996.6 (100) $[M - H]^-$; MALDI-TOF: 998.3 (10) $[M + H]^+$, 1020.3 (100) $[M + Na]^+$, 1036.3 (60) $[M + K]^+$; RP-HPLC: $R_t = 16.9$ min; LC-MS: $R_t = 13.0$ min.

H-Q(Trt)-A-C(Trt)-K(Boc)-OMe (7)

Fmoc-Lys(Boc)-OH (1.97 g, 4.21 mmol) was dissolved in a two-neck, round-bottomed flask in a mixture of DCM (9 ml) and DMF (4.5 ml). To this were added HOBt (569 mg, 4.21 mmol), DIC (651 μl, 4.21 mmol) and DMAP (51 mg, 0.42 mmol). The reaction mixture was stirred at r.t. for 30 min and then added to the pre-swollen resin. The reaction mixture was shaken at r.t. for 15 h, after which the resin was washed. Washing times were extended to 3 × 5 min. The reaction was repeated with 1 equivalent of amino acid and coupling reagents and 0.1 equivalents of DMAP for 6 h. The loading was determined by UV absorbance and was found to be 0.89 mmol/g, corresponding to a quantitative coupling yield. The other amino acids were coupled according to the following general procedure: The resin was treated with 2 × 20 ml of a solution of 20% piperidine in DMF for 2 × 20 min and washed. The TNBS test gave dark orange beads. Then the amino acid (2.47 g Fmoc-Cys(Trt)-OH, 1.31 g Fmoc-Ala-OH and 2.57 g Fmoc-Gln(Trt)-OH, 4.21 mmol), PyBOP (2.19 g, 4.21 mmol) and DIPEA (1.39 ml, 8.42 mmol) were added to the resin in DMF (20 ml). The reaction mixture was shaken at r.t. for 4 h. All TNBS tests gave colourless beads after one coupling. Final Fmoc deprotection was carried out with 2 × 20 ml of a solution of 20% piperidine in DMF for 2 × 20 min, followed by resin washing. For the cleavage, the resin (2.87 g, 0.49 mmol/g) was transferred to a round-bottomed flask equipped with a sintered glass filter and a three-way stopcock. It was suspended in MeOH (16 ml), Et_3N (8 ml) and DMF (16 ml). The resin was gently stirred at 50 °C for 24 h, after which the filtrate and washings were evaporated under reduced pressure. This cleavage was repeated twice to give a total mass of 583 mg of crude peptide **7** in a yield of 40%. The two first fractions of peptide **7** could be used without further purification. Exact Mass: 1046.5; ESI-MS: 1047.2 (60) $[M + H]^+$, 1069.3 (100) $[M + Na]^+$; RP-HPLC: $R_t = 19.1$ min; LC-MS: $R_t = 20.4$ min.

Coupling of 7 in Reverse Direction (8)

Resin **6** (70 mg, 0.17 mmol/g) was suspended in DMF (2.5 ml) and treated with HATU (15 mg, 40 μmol), 2,4,6-trimethylpyridine (11 μl, 80 μmol) and methyl ester **7** (41 mg, 40 μmol). The reaction mixture was flushed with argon and shaken at r.t. for 18 h, after which the mixture was washed. Exact Mass: 2026.0; TNBS test: colourless; NF31 test: colourless; MALDI-TOF: 1047.3 (65) (unidentified impurity), 1807.5 (100) $[M - Trt + Na]^+$, 2049.5 (90) $[M + Na]^+$; RP-HPLC: $R_t = 23.0$ min.

Methyl Ester, Fmoc and Acetate Deprotection (9)

Resin **8** (95 mg, 0.14 mmol/g) was treated with a solution of 0.33 M LiOH in MeOH/H_2O (97/3) at r.t. for 4 days, after which the resin was washed. Exact Mass: 1747.9; TNBS test: dark orange; NF31 test: dark red; MALDI-TOF: 1494.5 (65) (unidentified impurity); 1771.5 (100) $[M + Na]^+$, 1786.4 (35) $[M + K]^+$, 1793.4 (60) $[M - H + 2Na]^+$, 1808.4 (25) $[M - H + Na + K]^+$.

Backbone Cyclisation (10)

To a suspension of resin **9** (70 mg, 0.15 mmol/g) in DMF (2 ml) were added HOBt (27 mg, 0.20 mmol), TBTU (64 mg, 0.20 mmol) and DIPEA (60 μl, 0.35 mmol). The reaction mixture was shaken at r.t. overnight and was then washed. Exact Mass: 1729.9; TNBS test: colourless; NF31 test: colourless; ESI-MS: 1488.4 (20) $[M - Trt + H]^+$, 1730.2 (100) $[M + H]^+$, 1753.7 (15) $[M + Na]^+$; MALDI-TOF: 1508.4 (20) $[M - Trt + Na]^+$; 1753.3 (100) $[M + Na]^+$, 1769.3 (15) $[M + K]^+$; RP-HPLC: $R_t = 21.8$ min.

Side-chain Deprotection (11)

Resin **10** (35 mg, 0.15 mmol/g) was treated with a solution of TFA/H_2O/TIS (95/2.5/2.5) (1 ml) at r.t. for 2 h, after which the resin was washed. Exact Mass: 1145.6; MALDI-TOF: 1165.7 (100) $[M + Na]^+$; RP-HPLC: $R_t = 13.0$ min.

Preparative UV Cleavage (12)

Resin **11** (30 mg, 0.16 mmol/g) was suspended in a solution of 1% DMSO/dioxane (2 ml). Nitrogen was gently bubbled

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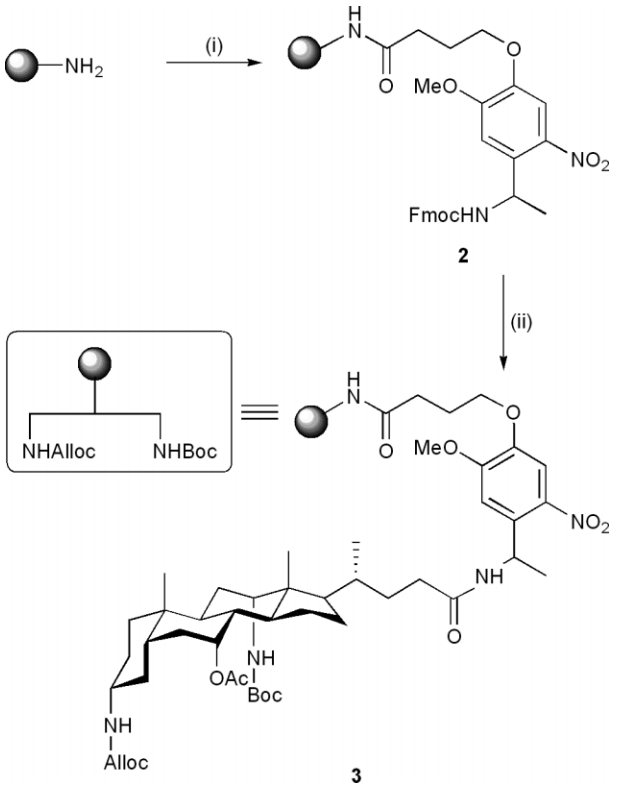
through the reaction vessel. Fresh dioxane (2 ml) was added whenever it had evaporated. The reaction vessel was treated with UV light at 365 nm for 4 h. The filtrate was collected and the resin was washed several times with dioxane. All solvents were lyophilised. The cleavage was repeated 4 times, giving the crude product **12** (5.3 mg, 47 μmol) in 88% yield. Exact Mass: 1145.6; MALDI-TOF: 1166.7 (100) $[M + Na]^+$, 1182.7 (30) $[M + K]^+$; RP-HPLC: $R_t = 13.0$ min.

RESULTS AND DISCUSSION

This study reports on the synthesis of a macrocyclic peptidosteroid in which the orthogonally protected bile-acid-based scaffold **1** was linked to a solid support via a photo-labile linker, allowing convenient reaction monitoring after each reaction step [15]. The scaffold amines were selectively deprotected, starting with the Boc-protected amine at C-12, which was then reacted with Fmoc-protected tetraglycine. Following Alloc deprotection and the attachment of a succinate linker in a one-pot transacylation step, a second tetrapeptide fragment, corresponding to part of the MV HNE sequence, was coupled via its free amine to the scaffold at C-3. The carboxylic ester was protected as a methyl ester during coupling. After saponification of the methyl ester and concomitant Fmoc removal, the two peptide chains were cyclised via their backbones, whereupon all side-chain-protecting groups were removed and the compound was cleaved from the solid phase.

In order to monitor every reaction step easily, a linker had to be used, which permitted convenient cleavage on a small scale under conditions orthogonal to all other reaction conditions. Therefore, we opted for the *o*-nitrobenzyl-based photo-labile linker developed by Holmes [16,17]. Coupling to TentaGel-NH_2 resin was effected using either DIC and HOBt or PyBOP and DIPEA, with a preference for the latter conditions due to shorter reaction times (Scheme 1). After Fmoc deprotection, scaffold **1** was coupled via its free carboxylic acid, preferably with PyBOP and DIPEA, though DIC and HOBt gave similar coupling yields after longer reaction times.

For peptide attachment, two possible strategies can be envisaged: (i) coupling of the entire peptide onto one scaffold amine, followed by cyclisation onto the other amine, or (ii) coupling of parts of the peptide to either scaffold-bound amine followed by cyclisation between the peptide termini. The first approach might prove difficult for longer sequences, and we therefore opted for the attachment of two peptide parts to either attachment point in an anti-parallel way. Considering that the C-12 position is most sterically hindered and since the acidic conditions required for Boc removal are not compatible with later peptide chain extension via Fmoc chemistry with acid-labile side-chain-protecting groups, the first tetrapeptide was attached to this



Scheme 1 Attachment of photo-labile linker and scaffold to the solid phase. (i) 4-{4-[1-(9-fluorenylmethoxycarbonylamino) ethyl]-2-methoxy-5-nitrophenoxy}butanoic acid, PyBOP, DIPEA, DMF, 2 h, r.t.; (ii) (a) 20% piperidine/DMF, 2 × 20 min, r.t.; (b) methyl 7-α-acetoxy-3α-[*N*-(allyloxycarbonyl)amino]-12α-[*N*-(*t*-butyloxycarbonyl)amino]-5β-cholan-24-oate (**1**), PyBOP, DIPEA, DMF, 2 h, r.t..

amine. Boc deprotection was achieved with a solution of 20% TFA in DCM. All reactions were monitored by the NF31 and TNBS colour tests [12–14] (see 'Materials and Methods' for more details). Boc deprotection could be monitored only with NF31, as sterical hindrance at C-12 prevents its reaction with TNBS [14]. HPLC analysis could not be used for the monitoring of these reactions, as the products do not absorb in the UV region. However, analysis by mass spectroscopy gave exclusively one product, as did the mass chromatogram from the LC-MS analysis.

In a following step, Fmoc-protected tetraglycine was coupled as a segment to the free amine in position 12 using DIC and HOAt (Scheme 2). This reaction was found to be strongly dependent on the peptide concentration rather than the equivalents used. A concentration of around 0.01 M gave a coupling yield of 100%, even when using only 1.5 equivalents of the peptide. Higher concentrations in contrast led to incomplete coupling, even when using up to 3 equivalents of the peptide. In these cases, the reaction had to be repeated to give complete coupling.

For the second peptide, a sequence from the actual epitope was chosen. In this way, the compatibility

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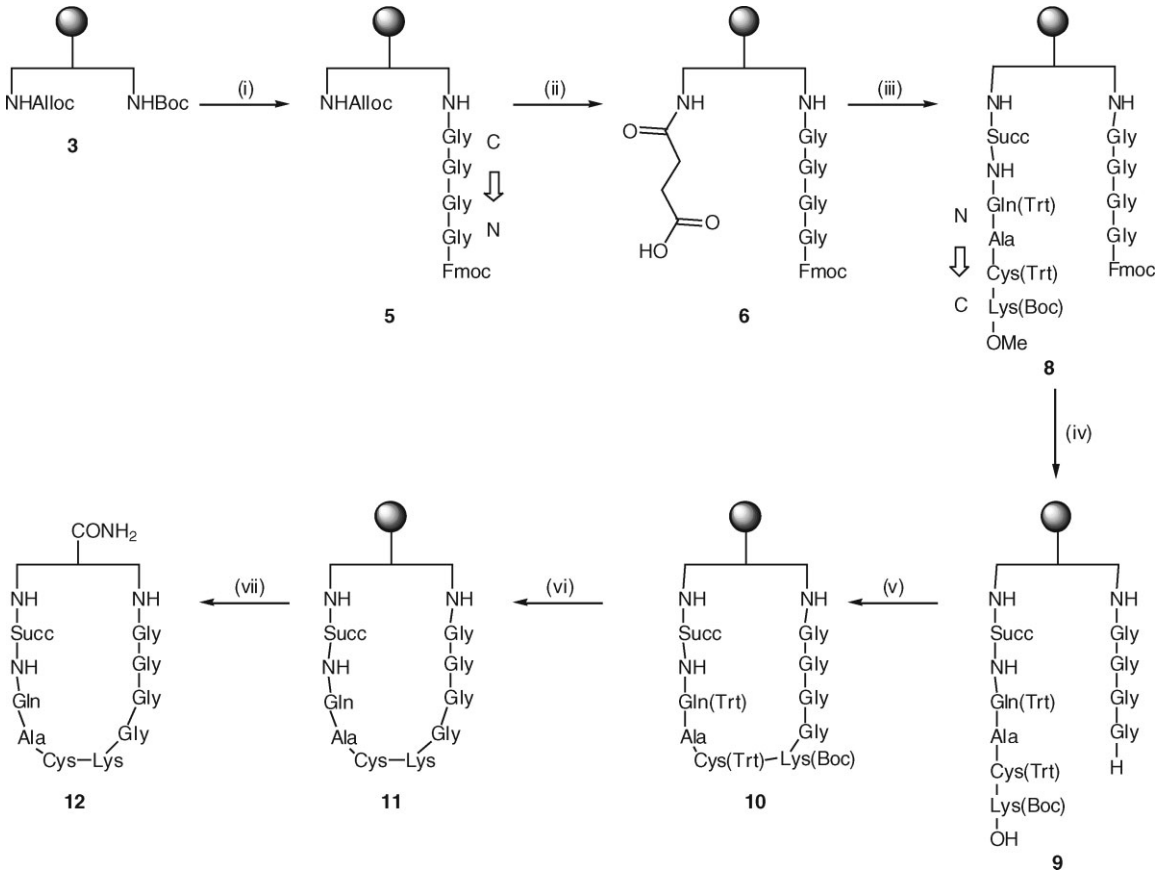

Scheme 2 Synthesis of the octapeptide–scaffold cycle. (i) (a) 20% TFA, DCM, 20 + 90 min, r.t.; (b) Fmoc-Gly_4-OH (**4**), HOAt, DIC, DMF, o/n, r.t.; (ii) $Pd(PPh_3)_4$, Bu_3SnH, succinic anhydride, DCM, 2 × 17 h, r.t.; (iii) H-Gln(Trt)-Ala-Cys(Trt)-Lys(Boc)-OMe (**7**), HATU, TMP, DMF, o/n, r.t.; (iv) LiOH 0.33M, MeOH/H_2O, 4 d, r.t.; (v) HOBt, TBTU, DIPEA, DMF, o/n, r.t.; (vi) TFA/H_2O/TIS (95/2.5/2.5), 2 h, r.t.; (vii) hν (365 nm), 1% DMSO/dioxane, 4 × 4 h, r.t. Succ: –CO–$(CH_2)_2$–CO–.

of the synthetic strategy with amino acid side-chain-protecting groups could be tested and protection of the terminal carboxylic acid of the tetrapeptide investigated. Also, the inclusion of a chiral amino acid at the *C*-terminus would allow monitoring of the extent of epimerisation during deprotection and cyclisation steps later on.

It is evident that the synthesis of a peptide loop–scaffold construct via the attachment of two smaller peptide fragments implies the coupling of one peptide strand via its carboxylic acid end in the classical C-to-N synthesis direction, whereas the second strand is linked to the scaffold via its terminal amine (Scheme 2). Although inverse solid-phase peptide synthesis (N-to-C strategy) is possible [18,19], it is much less popular because of the increased risk of epimerisation and the limited commercial availability of suitable amino acid building blocks. Convergent (sequential) peptide attachment [20,21], where the desired peptide is first synthesised on a separate solid phase, cleaved with its side chains protected, purified and subsequently attached to the scaffold, is a way of circumventing this limitation. However, also with convergent SPPS there remains an albeit reduced risk of epimerisation every time a carboxylic acid terminus containing a chiral amino acid is coupled or cyclised.

For this purpose, the amino acid sequence H-Gln(Trt)-Ala-Cys(Trt)-Lys(Boc)-OH was synthesized on Wang resin, using standard coupling procedures such as DIC and HOBt or the more time-efficient PyBOP and DIPEA. It was decided to cleave the resin from the solid phase as a methyl ester [22], as this avoids the awkward handling of highly apolar peptide in solution in an additional carboxylic acid protection step. Following final Fmoc deprotection, the resin was thus cleaved under basic conditions in a mixture of Et_3N, MeOH and DMF to afford the desired methyl ester H-Gln(Trt)-Ala-Cys(Trt)-Lys(Boc)-OMe (**7**).

In order to be able to attach the free amine of **7** to the scaffold-bound amine in position 3, a suitable linker had to be introduced. Succinic acid was found to be a potential handle to allow inversion of the peptide directionality. It is known from previous experiments that Alloc deprotection in an Fmoc-containing molecule such as **5** is not straightforward: In addition to the substantial risk of irreversible allylation of the deprotected amine even

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in the presence of scavengers, such compounds often undergo at least partial Fmoc deprotection under the influence of nucleophilic allyl scavengers [23]. In the case of compound **5**, it was found that Fmoc deprotection took place upon treatment of the resin with tetrakistriphenylphosphine palladium (0) ($Pd(PPh_3)_4$) and tributyltin hydride (Bu_3SnH). However, when a one-pot transacylation procedure adapted from solution synthesis [24] was used, only the desired product **6** was formed. For this purpose, resin **5** was reacted with an excess of succinic anhydride in the presence of Bu_3SnH and a catalytic amount of $Pd(PPh_3)_4$ in DCM. On most occasions, the reaction had to be repeated once to ensure full conversion of the starting material.

Tetrapeptide **7** was then coupled to the solid-phase-bound scaffold using HATU and TMP in DMF [25,26]. This time, a concentration of 0.05 M proved to give the highest coupling efficiency, whereas lower concentrations tended to give incomplete coupling. Disconcertingly, alongside the desired product **8**, the formation of a so-far-unidentified by-product of molecular weight 1024 was observed. This compound was also formed upon coupling with HOAt/DIC or PyBOP/DIPEA and could therefore not be related to a guanidinium-based side reaction. Fortunately, this impurity did not interfere in the subsequent reactions and was no longer detected after the cyclisation reaction.

The synthetic route was pursued with the saponification of the methyl ester on resin **8**. This was attempted with LiOH in dioxane and water [27–29], LiOH and $CaCl_2$ in water and isopropanol, sonicating for 3 h at 0 °C [30] or finally with bis(tri-*n*-butyltin) oxide (BBTO) in acetonitrile [31], but to no avail. Methyl ester deprotection was eventually achieved upon treatment of the resin with a 0.33 M solution of LiOH or NaOH in methanol/water (97/3) over several days [32]. These very harsh reaction conditions deprotected not only the methyl ester but also the Fmoc-protected amine and the acetate-protected alcohol in position 7 on the scaffold. However, we did not consider this as a major drawback of our synthetic strategy, as it is known that at least one α-oriented free hydroxyl group on bile-acid derivatives has shown to greatly improve ileal bile-acid absorption [33]. At this stage, it became impossible to analyse the reaction product via RP-HPLC, possibly owing to the very apolar character of the fully protected peptide strands contrasting with the zwitterionic character of the free termini. This meant that although the exclusive formation of resin **9** was observed by MALDI-TOF, no information was gained on the degree of epimerisation of this reaction. However, the group of Beck-Sickinger stipulates that in their comparable case, no degradation or racemisation products were observed [32, 34].

Head-to-tail cyclisation of the two peptide strands was carried using HOBt, TBTU and DIPEA in DMF [32]. Mass spectrometry showed that six equivalents of coupling reagents drove the reaction to completion without guanidinium formation, despite the presence of an excess of TBTU in the reaction mixture and no possibility for pre-activation. Moreover, no dimerisation through intermolecular reaction between peptide strands attached to different scaffolds was observed. This can be ascribed to the pseudo-dilution conditions under which resin-bound cyclisation reactions are carried out [35].

In a last step, the side-chain-protecting groups were removed with TFA/H_2O/TIS (95/2.5/2.5) to give compound **11**. Cleavage in 1% DMSO/dioxane under a UV lamp set at 365 nm gave the desired product **12** in a moderate cleavage yield of 40% [16,17].

CONCLUSIONS

A synthetic route towards a large cyclic peptidosteroid derivative has been developed. Through the introduction of a succinic acid linker on the scaffold 3 position, two peptide strands were coupled convergently in an anti-parallel way. Subsequent backbone cyclisation occurs smoothly, giving rise to a ring size equivalent to approximately 12 amino acids. The described synthetic strategy will be generally applicable to a variety of peptide sequences, thus representing a straightforward route to larger analogues, including an MV vaccine candidate containing the minimal HNE sequence.

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