Synthesis of cyclic peptide analogues of the 3_{10} helical Pro138-Gly144 segment of human aquaporin-4 by olefin metathesis†

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Four cyclic pentapeptides and two cyclic heptapeptides modelled on the 3_{10} helical Pro138-Gly144 segment of the water channel aquaporin-4 (AQP4) postulated to mediate adhesive interactions between AQP4 tetramers were synthesised by olefin metathesis. Three related acyclic pentapeptides Boc-Ser(All)-Xaa1-Val-Ser(All)-Gly-OMe (Xaa1 = Val, Aib; Boc = *tert*-butoxycarbonyl; All = allyl) and Boc-Ser(Bn)-Val-Val-Gly-Gly-OMe (Bn = benzyl) and two acyclic heptapeptides Boc-Pro-Pro-Ser(All)-Val-Val-Ser(All)-Gly-OMe and Boc-Pro-Pro-Ser(Bn)-Val-Val-Gly-Gly-OMe were also prepared. NMR, CD and IR data provided evidence that the peptides can access a 3_{10} helical structure in apolar solvents and pointed to a significant stabilising effect of the olefinic bridge on helicity in an aqueous environment. Thus we could demonstrate the viability of using ring closing olefin metathesis to stabilise short protein segments in the helical conformation that they adopt in their native protein environment. Our approach provides access to a set of peptides with potential binding affinity for AQP4.

Introduction

Aquaporins are water selective membrane channel proteins which allow rapid water transport across cell membranes at rates that can approach the diffusion limit.[1,2] So far 13 mammalian isoforms have been identified.[3] The isoform aquaporin-4 (AQP4) is the predominant water channel in the brain and is concentrated in astrocytic end-feet.[4,5] AQP4 plays an important role in the regulation of water homeostasis in the brain and in the pathophysiology of brain edema.[6] It has been demonstrated that mice lacking AQP4 or AQP4 anchoring proteins show less edema and suffer less damage to the brain in an ischemic stroke model than wild-type mice, validating AQP4 as a possible drug target for treatment of cerebral edema.[7–9]

Few inhibitors of aquaporin function are known and all are toxic, irreversible and/or unspecific.[10] This is true for the inorganic transition metal compounds, *e.g.* silver nitrate and mercurials, that are established aquaporin inhibitors.[11–13] In its correct orientation in the membrane AQP4 is not even inhibited by mercurials, hence its original name "mercury insensitive water channel".[14] A few reports have appeared on the inhibitory effect of small organic molecules like *p*-chloromercuri benzenesulfonate,[15] phloretin,[16,17] carbonic anhydrase inhibitors (of which acetazolamide is one example)[18,19] and quaternary ammonium salts.[20] However, these results have been challenged.[21,22] Thus, despite the range of important physiological and pathophysiological roles played by aquaporins,[1] we still lack specific and reversible inhibitors of any member of this class of channel proteins.

Searching for a way to approach the problem of developing AQP4 selective inhibitors we turned to the significant amount of structural information that has accumulated in recent years from electron diffraction (ED) and X-ray diffraction studies of aquaporins and aquaglyceroporins.[23–26] Recently, the first structure of AQP4 was determined by ED to 3.2 Å resolution.[27] The structure indicated that AQP4, like its isoform AQP0, may play a role in cell-cell adhesion.[27–29] Expressing AQP4 in L-cells, cells with no surface adhesive molecules, causes the cells to aggregate,[27] although this claim has recently been challenged.[30] The primary interaction between AQP4 molecules belonging to adjacent cells is mediated by loop C, part of which takes up a 3_{10} helical conformation.[27]

We have initiated a program to explore the potential of using synthetic peptides with similar primary and secondary structures to loop C as molecular scaffolds for side chains that could extend into the vestibule of the aquaporin and potentially block it. In the perivascular AQP4 pool (*i.e.* the AQP4 pool that is thought to constitute the main influx pathway for water during the development of brain edema) the targeted binding site is not engaged in binding to contiguous AQP4 tetramers and thus should be freely available for ligand binding.[31]

Inspection of the reported structure revealed that Pro139 and Val142 likely play important roles in mediating the interaction between two AQP4 tetramers.[27,29] Due to the mode of interaction (van der Waals/hydrophobic interactions and possibly non-classical C–H–O=C H-bonds[32]) it is likely of crucial importance that the conformations of the synthetic peptides very closely reproduce the conformation of the segment Pro139-Val142. Hence, it was deemed of great interest to explore methods that might afford

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† Electronic supplementary information (ESI) available: Synthetic procedures for compounds fully characterised in the literature, partial ROESY spectra of **29** and CD spectra recorded in MeOH. See DOI: 10.1039/b823559g

some stabilisation of the 3_{10} helical conformation of the segment outside of its natural protein environment.

Ring closing olefin metathesis (RCM) has been successfully applied to conformationally restrain peptides in a range of conformations.[33–35] Peptides rich in α-aminoisobutyric acid (Aib) residues are especially prone to form 3_{10} helices.[36,37] Recent work on Aib rich peptides have demonstrated the feasibility of stabilising a short peptide in a 3_{10} helical conformation by construction of a hydrocarbon bridge connecting the side chains of residues *i* and *i* + *3*, the key synthetic step being a ruthenium catalysed ring closing olefin metathesis reaction using Grubbs' catalysts.[38,39] The presence of a serine residue in loop C, *i.e.* Ser140, which could easily be allylated, and a Gly residue at the *i* + *3* position which did not seem to be involved in any key interactions made this an especially attractive strategy in the current project. However, to the best of our knowledge, this method had not been applied previously to short peptides containing only proteinogenic residues, which have a less pronounced tendency than disubstituted α-amino acids to adopt the required dihedral angles for a 3_{10} helix, or to 3_{10} helical protein segments. One of our main objectives is to demonstrate the applicability of this methodology to such cases, thus providing access to a set of conformationally restrained peptides with potential affinity for AQP4.

Several studies have demonstrated that diproline motifs often precede helices in protein structures and can serve as folding nuclei for short (3_{10}) helical peptides.[40,41] The presence of a Pro residue N-terminal to Pro139 in the relevant segment of AQP4 therefore strongly suggested including an additional Pro residue in the synthetic peptides despite this not being involved in the binding interaction. C-terminally to the Pro139-Val142 segment we chose to include two residues, one whose function would be to facilitate the construction of a bridge from the Ser residue, and a Gly representing the Gly144 residue. This choice struck a balance between including a sufficient number of residues to allow formation of a stable helix, keeping the molecular weight low and paying attention to the Lipinski Rule of Five,[42] and having a flexible arm (Gly) for later derivatisation. As a result four heptapeptides emerged as target compounds (Fig. 1).

Synthetic strategy

Because gram quantities of the various peptides were needed for further studies and derivatisation a solution phase protocol was adopted. The sequence similarity of the target heptapeptides suggested adopting a fragment condensation strategy based on the following retrosynthetic disconnections (Fig. 1).

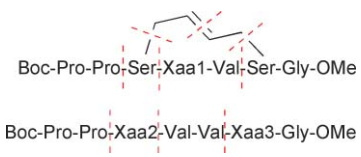


Fig. 1 Retrosynthetic disconnections. Cyclic heptapeptides: Xaa1 = Val, Aib; Acyclic heptapeptides: **31**: Xaa2 = Ser(All), Xaa3 = Ser(All); **33**: Xaa2 = Ser(Bn), Xaa3 = Gly.

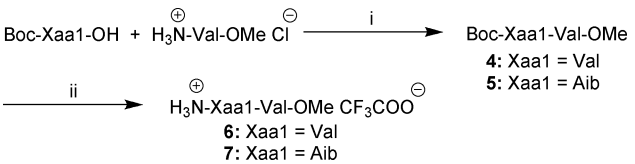
As the most convenient starting material for incorporation of the *O*-allyl L-serine residue appeared to be *N*-*tert*-butoxycarbonyl L-serine it seemed sensible to synthesise the fragments Boc-Ser(Bn)-Xaa1-Val-OMe and Boc-Ser(All)-Xaa1-Val-OMe rather than the fragments Boc-Pro-Pro-Ser(Bn)-OH and Boc-Pro-Pro-Ser(All)-OH. This left us with a choice between synthesising the N-terminal pentapeptidic fragments or the C-terminal pentapeptidic fragments indicated in Fig. 1. Because we initially did not expect to be able to do the RCM already at the pentapeptide stage both strategies seemed equally worthy of an attempt and it was decided to synthesise the N-terminal fragments first. It turned out that this strategy had to be revised in view of the problems incurred by saponification of these fragments (Scheme 2, see Results and Discussion) and the C-terminal pentapeptides were synthesised instead.

Results and discussion

Solution phase synthesis of pentapeptides

The dipeptide fragment Boc-Pro-Pro-OMe **1** common to all the target heptapeptides was synthesised in 82% yield by carbodiimide coupling using EDC/HOBt.‡ Deprotection of the C-terminus by saponification with lithium hydroxide in THF/H_2O (2:1) afforded the dipeptide Boc-Pro-Pro-OH **2** in 88% yield. The key building block Boc-*O*-allyl L-serine **3** was synthesised by analogy to a literature synthesis of Boc-*O*-benzyl L-serine by deprotonation with NaH (2.20 eq.) and allylation employing a small excess (1.10 eq.) of allyl bromide.[43] Yields, which varied between 60–83%, were generally comparable to literature values.[38,39]

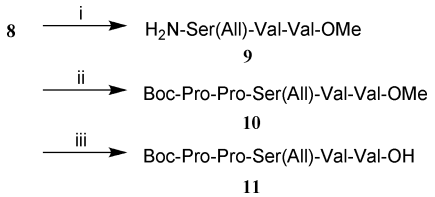
The dipeptides Boc-Val-Val-OMe **4** and Boc-Aib-Val-OMe **5** were synthesised in excellent yields (87% and 95% respectively) using EDC (1.10 eq.)/HOBt (1.00 eq.). Deprotection afforded the trifluoroacetates **6** and **7** in 97% and 82% yield respectively (Scheme 1).



Scheme 1 *Reagents and conditions:* **6**: (i) EDC, HOBt, DIPEA, DMF, rt, 21 h, 87%; (ii) TFA, CH_2Cl_2, rt, 2 h, 97%; **7**: (i) EDC, HOBt, DIPEA, DMF, 47 h, 95%; (ii) TFA, CH_2Cl_2, rt, 2 h, 82%.

The tripeptide **8** was synthesised by carbodiimide coupling in 95% yield (Scheme 4) and the N-terminus deprotected with neat formic acid. The resulting formate was not isolated, but converted to the free amine **9**. Carbodiimide coupling with **2** afforded pentapeptide **10** in 93% yield (Scheme 2). Disappointingly, saponification of **10** with NaOH in MeOH/H_2O (2:1) to form the free acid **11**, in analogy to a literature procedure[44] for hydrolysis of **1**, resulted in extensive epimerisation, as evidenced by the appearance of too many amide NH signals in the ^{1}H-NMR spectrum of the isolated product. This forced a revision of our

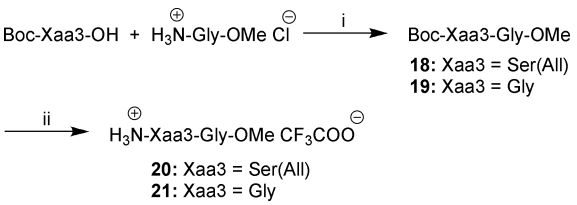
‡ HOBt: 1-hydroxybenzotriazole; EDC: *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide; PyBOP: (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; HATU: *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(7-azabenzotriazol-1-yl)uronium hexafluorophosphate



Scheme 2 *Reagents and conditions:* (i) a. HCOOH, rt, 9 h; b. K_2CO_3, H_2O, 82%; (ii) **2**, EDC, HOBt, DIPEA, DMF, 0 °C→rt, overnight, 93%; (iii) NaOH, MeOH/H_2O (2:1), rt, 8 h.

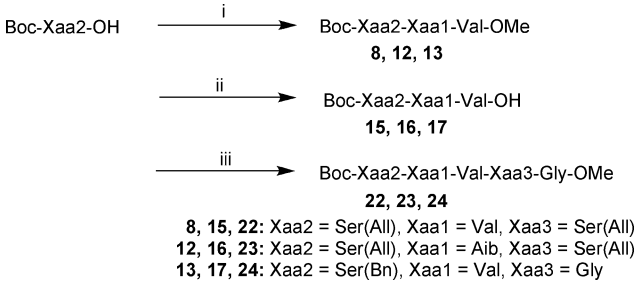
strategy and a decision was taken to synthesise the C-terminal pentapeptides (Fig. 1) instead.

Tripeptides **12** and **13** were synthesised analogously to **8** by condensation of **3** and **7** and *N-tert*-butoxycarbonyl *O*-benzyl L-serine **14** and **6** in 78% (sum of diastereomers) and 95% yield respectively (Scheme 4). The tripeptide **12** obtained was unfortunately quite heavily contaminated with its diastereomer and required recrystallisation twice from EtOAc/hexane (4:1) to afford a product stereoisomerically pure enough to be used in subsequent steps. To suppress epimerisation PyBOP‡ is a more preferred reagent for coupling to Aib residues.[45] However, due to the lower cost of EDC, the relatively large amounts of coupling reagent required and the simplicity of the purification procedure EDC was deemed acceptable. Initially, epimerisation during deprotection of the C-terminus of tripeptides **8**, **12** and **13** was problematic. Saponification of **8** using NaOH in MeOH/H_2O (2:1) at room temperature resulted in extensive (>30%) epimerisation. However, lowering the temperature to 0 °C, using a very slight excess of LiOH (1.10 eq.) in THF/H_2O and taking care to neutralise the excess LiOH with solid $NaHCO_3$ before work-up afforded the tripeptide **15** in acceptable yield (71%) and with only 2–3% epimerisation (Scheme 4). Likewise, tripeptide **16** was obtained in 88% yield under similar conditions and with comparable extent of epimerisation (Scheme 4). Tripeptide **17** on the other hand was isolated in anomalously low yield (42%) and had to be purified by dissolving the crude product in EtOAc and precipitating it by addition of hexane (Scheme 4). The C-terminal dipeptides **18** and **19** were synthesised by the carbodiimide method in 84% and 70% yield respectively and their N-termini deprotected with 50% TFA in CH_2Cl_2 to afford the trifluoroacetates **20** and **21** (Scheme 3) as viscous oils.



Scheme 3 *Reagents and conditions:* **20**: (i) EDC, HOBt, DIPEA, DMF, rt, 24 h, 84%; (ii) TFA, CH_2Cl_2, rt, 2 h, 98%; **21**: (i) EDC, HOBt, DIPEA, DMF, rt, 15 h, 70%; (ii) TFA, CH_2Cl_2, rt, 2 h, quant.

Condensation of tripeptide **15** with the dipeptide **20** in CH_2Cl_2 afforded the pentapeptide **22** in 89% yield (Scheme 4, sum of diastereomers). The carbodiimide coupling was somewhat hampered by 10–20% epimerisation. Other coupling reagents, *e.g.* HATU‡, might have suppressed epimerisation more efficiently,[46] but the much lower cost of EDC made this more attractive in a large-scale solution phase preparation. Fortunately, it turned out to be possible to obtain stereoisomerically pure (97–98%) material by recrystallisation from absolute EtOH. The pentapeptides **23** and **24** were obtained analogously to **22** in 73% and 78% yield respectively and with less epimerisation (Scheme 4).



Scheme 4 *Reagents and conditions:* **22**: (i) **6**, EDC, HOBt, DIPEA, DMF, 0 °C→rt, 22 h, 94%; (ii) LiOH, THF/H_2O (2:1), 0 °C, 3 h, 71%; (iii) **20**, EDC, HOBt, DIPEA, CH_2Cl_2, 0 °C→rt, 16 h, 89%; **23**: (i) **7**, EDC, HOBt, DIPEA, DMF, 0 °C→rt, 22 h, 78% (sum of diastereomers); (ii) LiOH, THF/H_2O (2:1), 0 °C, 3 h, 88%; (iii) **20**, EDC, HOBt, DIPEA, CH_2Cl_2, 0 °C→rt, 27 h, 73%; **24**: (i) **6**, EDC, HOBt, DIPEA, DMF, 0 °C→rt, overnight, 95%; (ii) LiOH, THF/H_2O (2:1), 0 °C, 3 h, 42%; (iii) **21**, EDC, HOBt, DIPEA, CH_2Cl_2, 0 °C→rt, 24 h, 78%.

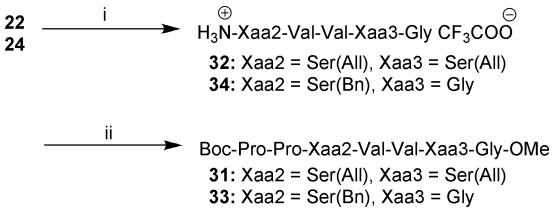
Pentapeptide **22** was found to have special properties. In contrast to **23** and **24** it precipitated from the reaction mixture and was surprisingly poorly soluble in both CH_2Cl_2 and EtOAc. Aqueous work-up consistently resulted in a troublesome emulsion. Because of the poor solubility in the organic phase the suspension of **22** had to be dried with 3 Å molecular sieves before removal of the solvent. Interestingly, direct injection of a solution of **22** in 0.5% (v/v) aqueous DMSO into a mass spectrometer demonstrated that dimers and, to a lesser extent, trimers were present even in the gas phase at 365 °C, suggesting a quite strong interaction between the monomers. All pentapeptides were synthesised on a multigram scale to allow for the possibility of constructing a small library based on the target heptapeptides as scaffolds. As an example, a total of 17 g of the pentapeptide **22** was synthesised.

Ring closing olefin metathesis

At high dilution (~4 mM) **22** dissolved in CH_2Cl_2 and was cyclised to **25** in 41% yield using Grubbs' 2nd generation catalyst[47] (20 mol%) (Scheme 6). The catalyst was added in two equally large portions, with the second portion being added after 2–3 hours. The E/Z selectivity was estimated to 14:1 from the ^{1}H-NMR spectrum, which compares nicely with previous work on Aib rich peptides where a 20:1 E/Z selectivity was observed.[38] The successful cyclisation suggests that **22** can access a helical conformation in solution. However, initially, purification of **25** from unreacted **22** by flash chromatography posed a serious problem. To minimise problems with purification it was therefore essential that the RCM was run under conditions which maximised the yield of cyclic peptide. The pentapeptide **23** was similarly cyclised to yield the cyclic product **26** (Scheme 6). However, the yield was higher (52%) and lower catalyst loadings (15 mol%; 10 mol% + 5 mol% after 1–2 hours) were required.

Synthesis of heptapeptides

The cyclic pentapeptides **25** and **26** were deprotected to give the trifluoroacetates **27** and **28** in 95% and 71% yield, respectively. Subsequent coupling with **2** using the EDC/HOBt method afforded the heptapeptides **29** and **30** in 78% and 82% yield, respectively (Scheme 6). Also the acyclic analogue **31** was synthesised in order to allow a study of possible conformational changes induced on cyclisation, but, most importantly, for its general utility as a possible AQP4 ligand in itself. Condensation of **2** and **32** provided this heptapeptide in 73% yield (Scheme 5). The trifluoroacetate **32** was obtained in 96% yield from **22** by treatment with 50% TFA in CH_2Cl_2. Finally, the heptapeptide **33**, corresponding to the native sequence of the relevant 3_{10} helical segment of AQP4, was synthesised analogously from trifluoroacetate **34** and **2** by way of carbodiimide coupling (Scheme 5).



Scheme 5 *Reagents and conditions:* **33**: (i) TFA, CH_2Cl_2, rt, 1 h 30 min, 92%; (ii) **2**, EDC, HOBt, CH_2Cl_2, 0 °C→rt, 23 h, 78%; **31**: (i) TFA, CH_2Cl_2, rt, 1 h 30 min, 96%; (ii) **2**, EDC; HOBt, DIPEA, CH_2Cl_2, rt, 21 h, 73%.

The trifluoroacetate **34** was obtained from its N-protected precursor **24** in 92% yield. The protecting groups were not removed from the final products to avoid potential problems with epimerisation on saponification of the C-terminus as seen for the shorter peptides and because some evidence points towards the Boc group having an important influence on the ability of small peptides to adopt structured conformations in solution.[48]

All pentapeptidic trifluoroacetates were efficiently purified from excess TFA by washing with Et_2O and were obtained as white powders.

Structural studies

CD measurements

Circular dichroism (CD) spectra are commonly used to determine the presence of different secondary structure elements in peptides and proteins. Far-UV CD spectra of several of the neutral penta- and heptapeptides were recorded in the structuring solvent 2,2,2-trifluoroethanol (TFE) (Figs. 2 and 3), MeOH† and H_2O/MeOH (39:1) (Fig. 4). In TFE all the spectra, with one exception, displayed a strong negative minimum at 202–203 nm corresponding to the π–π* transition and a much weaker negative minimum at 218–222 nm corresponding to the n-π* transition. In addition, the ratio $R = \Theta_{218-222}/\Theta_{202-203}$ was found to be approximately 0.3 for all pentapeptides but **26** (0.65) and approximately

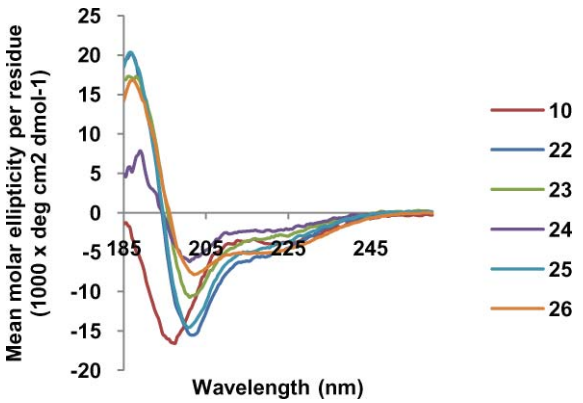
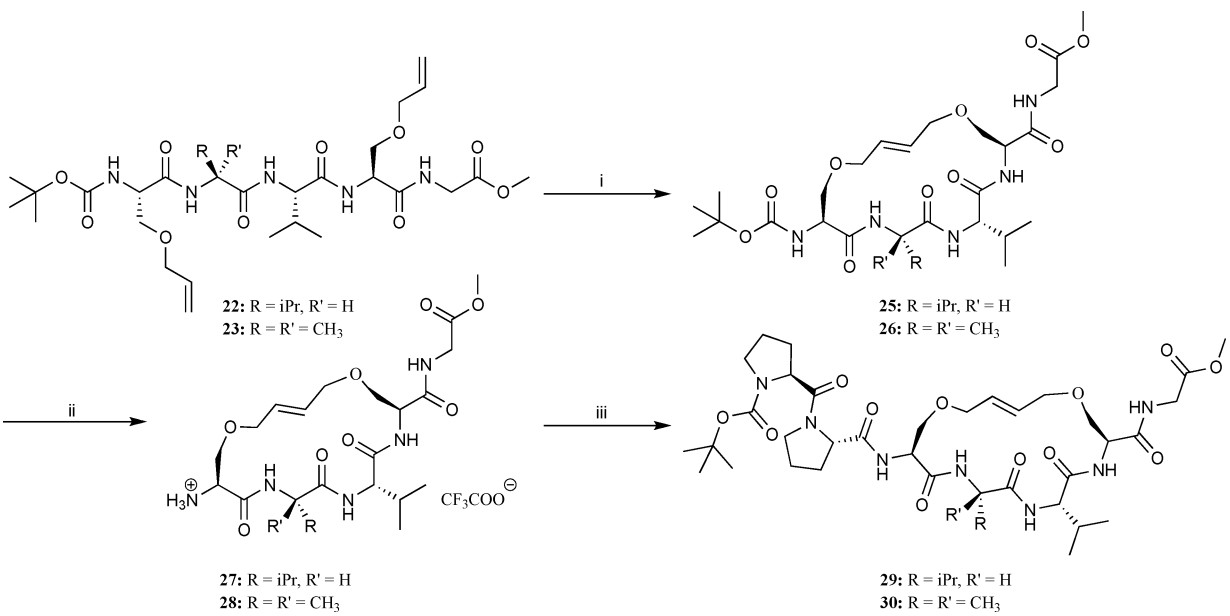


Fig. 2 CD spectra of some of the synthesised pentapeptides in TFE.



Scheme 6 *Reagents and conditions:* **29**: (i) Grubbs' 2nd generation catalyst (20 mol%), CH_2Cl_2, rt, 7 h 30 min, 42%; (ii) TFA, CH_2Cl_2, rt, 2 h, 95%; (iii) **2**, EDC, HOBt, DIPEA, CH_2Cl_2, 0 °C→rt, 22 h, 78%; **30**: (i) Grubbs' 2nd generation catalyst (15 mol%), CH_2Cl_2, rt, 6 h, 52%; (ii) TFA, CH_2Cl_2, rt, 1 h, 71%; (iii) **6**, EDC, HOBt, DIPEA, CH_2Cl_2, 0 °C→rt, 22 h, 82%.

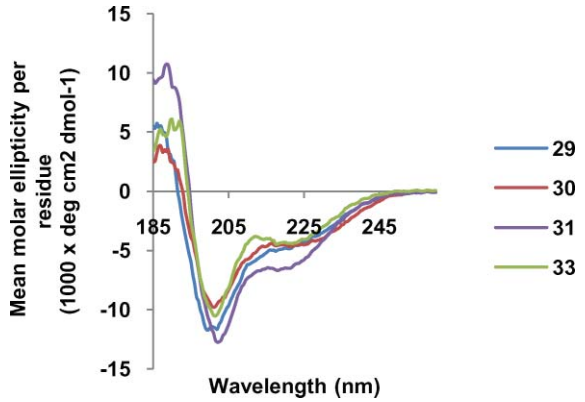


Fig. 3 CD spectra of the synthesised heptapeptides in TFE.

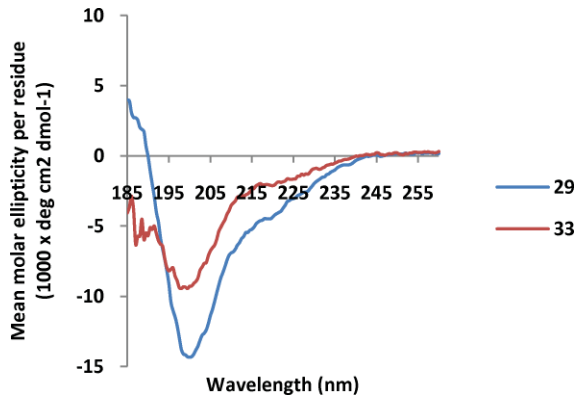


Fig. 4 CD spectra of the heptapeptides **29** and **33** in H_2O/MeOH (39:1).

0.4–0.5 for all heptapeptides. Similar values have been reported to be characteristic of short 3_{10}-helical peptides.[39]

The spectrum of **10** on the other hand displayed a minimum at 197.5 nm and a very weak negative maximum at 215–217 nm, which clearly demonstrated a much larger contribution from random coil conformations (negative minimum at 195 nm and weakly positive maximum at 212 nm). Similar spectra were obtained in MeOH, with one notable exception for the acyclic heptapeptide **33**, whose strongest minimum was significantly broadened. Further, the R value changed from approximately 0.4 in TFE to approximately 0.25 in MeOH. These results indicated that the contribution from helical conformations might be considerably smaller for the acyclic peptide **33** in a less structuring solvent. This was corroborated by our findings in a predominantly aqueous environment consisting of water/methanol (39:1) (Fig. 4).

Whereas the CD spectrum of **29** still resembled that in TFE and was similar to the reported signature spectrum of a 3_{10} helix in water[49] the changes were much more dramatic for **33** and thus provided a compelling indication that the olefinic bridge may stabilise the helical structure of **29** in aqueous solution.

IR measurements

The 3_{10} helix is defined by intramolecular $i \rightarrow i + 3$ H-bonds. Peptides adopting a helical conformation in solution should therefore show an absorption band in their IR spectra in the range 3300–3400 cm^{-1}, indicative of H-bonded amide NHs. The IR spectrum of the cyclic pentapeptide **25** in $CDCl_3$ shows two distinct NH stretching bands at 3426 cm^{-1} and 3345 cm^{-1}, respectively, of roughly similar intensities, with the latter band indicating the presence of H-bonding. Because only 2 out of 5 NH groups can take part in intramolecular H-bonding in a pentapeptide with 3_{10} helical structure the observed IR bands also supports the possibility that **25** can adopt a helical structure in $CDCl_3$.

NMR studies

For the dipeptide and tripeptide products only 1D spectra were recorded, whereas for all the penta- and heptapeptides 2D COSY and 2D TOCSY spectra were recorded in addition to aid residue specific assignment of the proton resonances. For the heptapeptides **29** and **30** sequence specific information was obtained from 2D ROESY spectra. In general, the ^{1}H-NMR spectra of the peptides containing a diproline moiety were complex. *Cis*/*trans* isomerism around the carbamate bond and between two proline residues is a well-known phenomenon.[41,50,51] This flexibility together with the presence of 14 unique proton types only in the proline residues, of which 12 have similar chemical shifts, and the presence of both (E)- and (Z)-isomers for the cyclic products all contributed to the observed complexity of the spectra. However, there were variations in the spectral complexity between the proline containing products. For example, the ^{1}H-NMR spectrum of heptapeptide **30** had a simpler appearance than that of heptapeptide **29**, especially evident in the NH region. This could be taken as an indication of a higher degree of structural order in the former. The 2D ROESY spectrum of heptapeptide **30** in CD_2Cl_2 clearly demonstrated the presence of all possible NH(*i*)→NH(*i* + *1*) ROEs, which is indicative of a helical peptide (Fig. 5). These ROEs are not specific for a 3_{10}-helix, but are also seen in α-helical peptides.[41,52,53] However, all possible $C^{\alpha}H(i)\rightarrow NH(i + 2)$ ROEs, which are diagnostic for 3_{10}-helices, were also observed, with the exception of a possible $C^{\alpha}H(Val)\rightarrow NH(Gly)$ ROE, which was hard to distinguish due to overlap (Fig. 6).[41,52,53] Finally, two of three possible $C^{\alpha}H(i)\rightarrow NH(i + 3)$ correlations, typical of mixed α-/3_{10}-helical conformations, were observed. Overlap with a more intense peak again precluded a possible $C^{\alpha}H(Ser(All)_1)\rightarrow NH(Ser(All)_2)$ ROE from being distinguishable. Evidence for a 3_{10}-helical conformation in CD_2Cl_2 could also be found in the 2D ROESY spectrum of **29**. This included three short-range NH(*i*)→NH(*i* + *1*), four medium-range $C^{\alpha}H(i)\rightarrow NH(i + 2)$ and two long-range $C^{\alpha}H(i)\rightarrow NH(i + 3)$ cross-peaks respectively.† Possible $NH(Ser(All)_2)\rightarrow NH(Gly)$, $C^{\alpha}H(Val_2)\rightarrow NH(Gly)$ and the two $C^{\alpha}H(Ser(All)_1)\rightarrow NH(Ser(All)_2)$ and $C^{\alpha}H(Val_1)\rightarrow NH(Gly)$ cross-peaks were not discernible due to overlap. In summary, the 2D ROESY data support the notion that both **29** and **30** can access a 3_{10}-helical conformation in CD_2Cl_2. That neither peptide can be perfectly helical is, however, seen by the presence of some additional $C^{\alpha}H(i)\rightarrow NH(i + 1)$ ROEs. Finally, the intensity of the $C^{\alpha}H(Pro_1)\rightarrow NH(Ser(All)_1)$ ROE is smaller for **29** than in the case of **30**. This may be taken in support of the hypothesis that the proline residues of heptapeptide **29** are conformationally more disordered than in **30**.

Conclusions

In summary the pentapeptides **10**, **22–26** and heptapeptides **29–31** and **33**, which are of potential significance for the development of AQP4 inhibitors, have been synthesised by standard

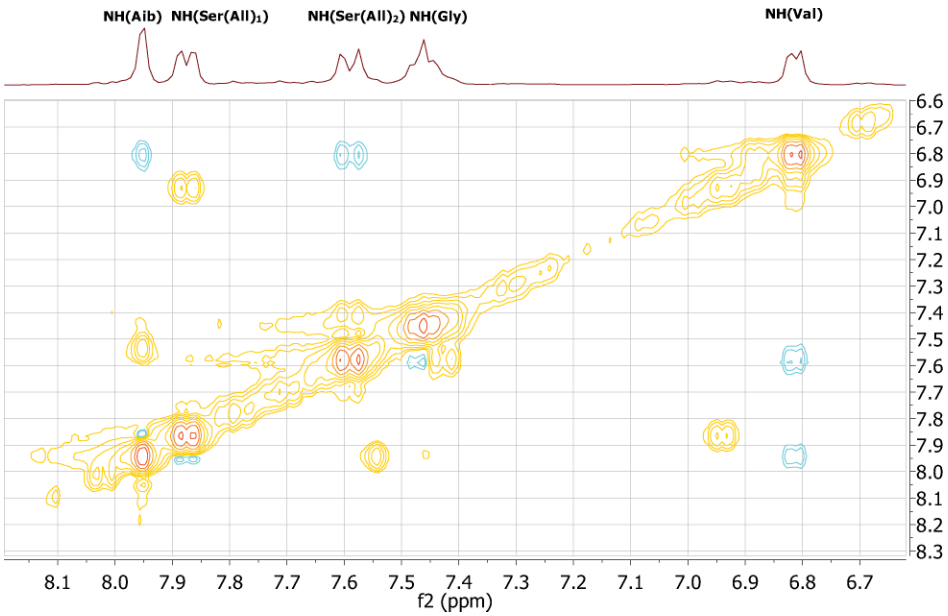


Fig. 5 Partial ROESY spectrum of **30** displaying NH(*i*)→NH(*i* + *1*) ROEs.

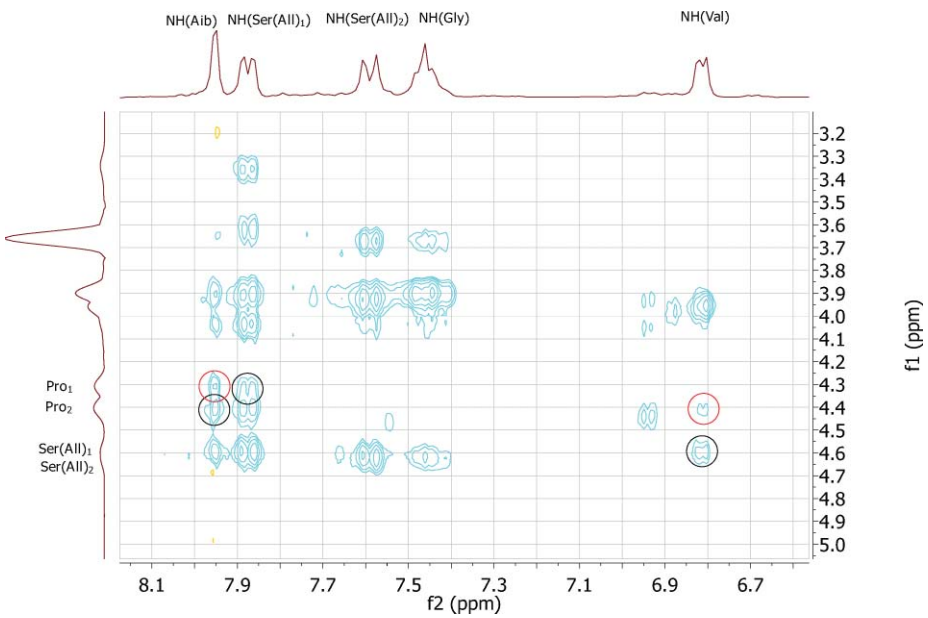


Fig. 6 Partial ROESY spectrum of **30** displaying $C^{\alpha}H(i)\rightarrow NH(i + 2)$ and $C^{\alpha}H(i)\rightarrow NH(i + 3)$ cross-peaks (black and red circles, respectively).

solution phase techniques and ring-closing metathesis. We have thus demonstrated the feasibility of scaling up and extending the reported methodology on Aib rich peptides[38,39] to peptides containing only proteinogenic amino acids (*i.e.* with the obvious exception of the *O*-allyl L-seryl residue, which is intrinsically required in order to be able to employ the methodology) and to protein segments. Despite quite serious initial problems with epimerisation, in particular of tripeptides **8**, **12** and **13** during saponification of the methyl ester functionality, these challenges have been addressed and the target penta- and heptapeptides have been obtained in near stereoisomerically pure form on multigram and gram scale, respectively. It has been demonstrated by CD, IR and 2D ROESY experiments that there is a significant contribution from a 3_{10} helix in the ensemble of conformations available to the peptides in apolar, structuring solvents like TFE and CD_2Cl_2, supporting the secondary structure assigned to this segment in the low-resolution ED structure of AQP4.[27] Most importantly, however, the CD spectra of **29** and **33** in H_2O point to the olefinic bridge having a stabilising effect on the helical conformation of the protein segment in a realistic environment for an AQP4 inhibitor. Potential applications of the methodology unrelated to AQP4 includes stabilisation of the 3_{10} helical conformation of a peptide corresponding to the N-terminal residues of the PB1 subunit of influenza virus RNA polymerase, an element recently identified as responsible for an essential subunit interaction and a potential lead structure for development of new influenza drugs.[54]

Future work will focus on further structural elucidation of the heptapeptides and establishing the affinity of the peptides for AQP4 in a radioligand binding assay.

Experimental

General

Chemicals were purchased from Sigma-Aldrich Co. and used as received unless otherwise stated. All solvents were of HPLC quality and all reagents were more than 98% pure. Flash chromatography was carried out using Silica Gel 60 (particle size: 0.04–0.063 mm/230–400 mesh) from Aldrich Co. NMR spectra were recorded in $CDCl_3$, d_6-DMSO or CD_2Cl_2 on a Bruker Avance DPX200 or a Bruker Avance DPX300 instrument at 200 MHz and 300 MHz respectively. All 2D spectra were recorded in phase sensitive mode using the TPPI (time proportional phase incrementation) method. The spectra were calibrated against internal tetramethylsilane, residual $CHCl_3$ ($\delta_H = 7.26$ ppm, $\delta_C = 77.0$ ppm), $CHDCl_2$ ($\delta_H = 5.32$ ppm, $\delta_C = 54.00$ ppm) or CHD_2SOCD_3 ($\delta_H = 2.50$ ppm, $\delta_C = 39.43$ ppm). For all compounds, with the exceptions of **29** and **30**, only residue specific assignments were made. Three-letter amino acid abbreviations are given in parenthesis where the assignment of signals otherwise could be ambiguous. The residue *O*-allyl L-serine has been abbreviated *Ser(All)* and the residue *O*-benzyl L-serine as *Ser(Bn)*. Identical residues, *e.g.* two valines, are numbered starting from the N-terminus. For the cyclic penta- and heptapeptides the chemical shifts given are for the major stereoisomer. Coupling constant (*J*) values are rounded off to the nearest whole number and given in Hz. Levels of epimerisation were estimated from the ratios of the N*H* integrals in the ^{1}H-NMR spectra. High-resolution mass spectrometric analyses were carried out on a Micromass Q-Tof-2 instrument with electrospray ionisation. CD spectra were measured on a JASCO J-810 spectropolarimeter between 185 and 260 nm using a 0.1 cm pathlength quartz cell and recording one data point every 0.5 nm. All spectra were baseline corrected, averaged over 5 scans, converted to a uniform scale of mean molar ellipticity per residue (in $10^3 \times$ deg cm^2 dmol^{-1}) and plotted using Microsoft Excel. The temperature was maintained at 20 °C and the concentrations of all peptides were in the range of 200–300 μM. IR spectra were recorded on a Perkin Elmer Spectrum BX FTIR instrument.

Synthetic procedures†

L-Valyl L-valine methyl ester trifluoroacetate (6). *N-tert*-Butoxycarbonyl L-valyl L-valine methyl ester **4** (10.07 g, 30.48 mmol) was treated with a 50% (v/v) TFA solution in CH_2Cl_2 for 2 hours at room temperature. The solvent and bulk of excess TFA were evaporated affording a clear, crystalline solid which contained TFA as judged by ^{1}H-NMR. Small portions of purified $CHCl_3$ (*i.e.* without EtOH as stabiliser) were added and evaporated under reduced pressure four times resulting in an off-white solid. The solid was washed with Et_2O (3 × 50 mL) and dried under reduced pressure affording a white solid (10.20 g, 97%). The compound has been mentioned in the literature, but spectral data was not provided[55]; δ_H (300 MHz; d_6-DMSO) 8.62 (1H, d, *J* 7, N*H*), 8.17 (3H, br s, NH_3^+), 4.19 (1H, dd, *J* 7 and 6, $C^{\alpha}H(Val_2)$), 3.75 (1H, br d, *J* 5, $C^{\alpha}H(Val_1)$), 3.64 (3H, s, OCH_3), 2.16–2.01 (2H, m, $CH(CH_3)_2$), 0.96–0.90 (12H, m, $CH(CH_3)_2$); δ_C (75 MHz; d_6-DMSO) 171.4, 168.4, 158.4 (q, J_{CF} 31), 117.1 (q, J_{CF} 297), 57.7, 56.9, 51.7, 29.9, 29.6, 18.8, 18.1, 18.1, 17.4; *m/z* (ESI) 231.1710 (M^+; $C_{11}H_{23}N_2O_3$ requires 231.1708).

α,α-Dimethylglycyl L-valine methyl ester trifluoroacetate (7). A 50% (v/v) solution of TFA in CH_2Cl_2 (350 mL) was added to *N-tert*-butoxycarbonyl α,α-dimethylglycyl L-valine methyl ester **5** (33.50 g, 0.1059 mol) at room temperature and the reaction mixture stirred for 2 hours. The solvent and bulk of excess TFA were removed under reduced pressure. The residue was washed with Et_2O (3 × 200 mL) and dried under reduced pressure affording a clear oil. Dichloromethane (3 × 200 mL) was added and evaporated under reduced pressure affording a sticky white solid (28.51 g, 82%); δ_H (300 MHz; d_6-DMSO) 8.42 (1H, d, *J* 8, N*H*), 8.26 (3H, br s, NH_3^+), 4.17 (1H, dd, *J* 8 and 8, $C^{\alpha}H(Val)$), 3.64 (3H, s, OCH_3), 2.16–2.05 (1H, m, $CH(CH_3)_2$), 1.52 (3H, s, $CH_3(Aib)$), 1.50 (3H, s, $CH_3(Aib)$), 0.91 (3H, d, *J* 6, $CH_3(Val)$), 0.88 (3H, d, *J* 6, $CH_3(Val)$); δ_C (75 MHz; d_6-DMSO) 172.0, 171.6, 158.1 (q, J_{CF} 32), 116.9 (q, J_{CF} 297), 58.3, 56.5, 51.7, 29.5, 23.2, 23.1, 19.0, 18.6; *m/z* (ESI) 217.1556 (M^+; $C_{10}H_{21}N_2O_3$ requires 217.1552).

***N-tert*-Butoxycarbonyl *O*-allyl L-seryl L-valyl L-valine methyl ester (8).** *N-tert*-Butoxycarbonyl *O*-allyl L-serine **3** (11.31 g, 46.10 mmol) was dissolved in DMF (40 mL). A solution of L-valyl L-valine methyl ester trifluoroacetate **6** (15.87 g, 46.09 mmol) and *N*,*N*-diisopropylethylamine (5.96 g, 46.1 mmol) in DMF (60 mL) was added in one portion and the solution cooled to 0 °C (ice bath). HOBt hydrate (7.06 g, 46.1 mmol) was added. Finally, EDC hydrochloride (9.72 g, 50.7 mmol) was added together with 30 mL DMF. The reaction mixture was stirred for 22 hours before the solvent was evaporated. The residue was taken up in EtOAc (250 mL) and washed with 2 M aqueous H_2SO_4 (3 × 100 mL), 7.5% (w/w) aqueous K_2CO_3 (3 × 100 mL) and saturated brine (100 mL). The solution was dried with anhydrous $MgSO_4$ and the solvent evaporated affording an off-white solid (19.82 g, 94%); δ_H (300 MHz; d_6-DMSO) 8.18 (1H, d, *J* 8, $NH(Val_x)$), 7.62 (1H, d, *J* 9, $NH(Val_y)$), 7.03 (1H, d, *J* 8, NH(Ser(All))), 5.83 (1H, ddt, *J* 5, 10 and 17, $CH{=}CH_2$), 5.23 (1H, ddt, *J* 1, 3 and 17, $CH{=}CHH$), 5.11 (1H, ddt, *J* 1, 3 and 10, $CH{=}CHH$), 4.34 (1H, dd, *J* 7 and 9, $C^{\alpha}H(Val_y)$), 4.22–4.17 (1H, m, $C^{\alpha}H$(Ser(All))), 4.13 (1H, dd, *J* 6 and 8, $C^{\alpha}H(Val_x)$), 3.92 (2H, dt, *J* 1 and 5, $CH_2CH{=}CH_2$), 3.61 (3H, s, OCH_3), 3.53–3.49 (2H, m, CH_2), 2.11–1.86 (2H, m, $CH(CH_3)_2$), 1.38 (9H, s, $(CH_3)_3$), 0.90–0.79 (12H, m, CH_3); δ_C (75 MHz; d_6-DMSO) 171.6, 171.0, 169.5, 155.1, 134.9, 116.3, 78.2, 70.9, 69.5, 57.4, 56.8, 54.5, 51.4, 31.1, 29.5, 28.0, 18.9, 18.8, 18.1, 17.7; *m/z* (ESI) 480.2666 ([M + Na]$^+$; $C_{22}H_{39}N_3O_7Na$ requires 480.2685).

***O*-Allyl L-seryl L-valyl L-valine methyl ester (9).** *N-tert*-Butoxycarbonyl *O*-allyl L-seryl L-valyl L-valine methyl ester **8** (6.80 g, 14.9 mmol) was stirred in formic acid (70 mL) at room temperature for 9 hours and the formic acid evaporated. The residue was diluted with water (110 mL) and the aqueous solution washed with Et_2O (3 × 30 mL). The pH was adjusted to pH 9 by addition of solid K_2CO_3 and the resulting solution extracted with EtOAc (3 × 55 mL). The combined organic extracts were washed with saturated brine (30 mL) and dried with anhydrous $MgSO_4$.

The solvent was evaporated affording a white solid (4.35 g, 82%); δ_H (200 MHz; d_6-DMSO) 8.26 (1H, d, *J* 8, N*H*(Val_x)), 8.03 (1H, d, *J* 9, N*H*(Val_y)), 5.85 (1H, ddt, *J* 5, 10 and 17, C*H*=CH_2)), 5.24 (1H, ddt, *J* 1, 3 and 17, CH=CH*H*), 5.12 (1H, ddt, *J* 1, 3 and 10, CH=C*H*H), 4.36 (1H, dd, *J* 6 and 9, $C^{\alpha}H$(Val_y)), 4.12 (1H, dd, *J* 6 and 8, $C^{\alpha}H$(Val_x)), 3.93 (2H, dt, *J* 1 and 5, CH_2CH=CH_2), 3.61 (3H, s, OCH_3), 3.49 (2H, d, *J* 5, CH_2), 3.43–3.39 (1H, m, $C^{\alpha}H$(Ser(All))), 2.38 (2H, br s, NH_2), 2.12–1.86 (2H, m, C*H*(CH_3)$_2$), 0.91–0.78 (12H, m, CH_3).

***N-tert*-Butoxycarbonyl L-prolyl L-prolyl *O*-allyl L-seryl L-valyl L-valine methyl ester (10).** *N-tert*-Butoxycarbonyl L-prolyl L-proline **2** (3.80 g, 12.2 mmol) and *O*-allyl L-seryl L-valyl L-valine methyl ester **9** (4.35 g, 12.2 mmol) were dissolved in DMF (25 mL) and the solution cooled to 0 °C. HOBt hydrate (1.87 g, 12.2 mmol) was added under stirring. EDC hydrochloride (2.57 g, 13.4 mmol) was added to the reaction mixture in small portions. The reaction mixture was stirred overnight and the solvent evaporated. The residue was taken up in EtOAc (50 mL) and washed with 2 M HCl (3 × 25 mL), 7.5% (w/w) K_2CO_3 (3 × 25 mL) and saturated brine (20 mL). The solution was dried with anhydrous $MgSO_4$ and the solvent evaporated affording the title compound as a white solid (7.34 g, 93%); δ_H (300 MHz; d_6-DMSO) 8.10 (1H, d, *J* 8, N*H*(Val_x)), 7.98 (1H, d, *J* 8, N*H*(Ser(All))), 7.60 (1H, d, *J* 9, N*H*(Val_y), rotamer 1), 7.59 (1H, d, *J* 9, N*H*(Val_y), rotamer 2), 5.83 (1H, ddt, *J* 5, 11 and 17, C*H*=CH_2), 5.24 (1H, ddt, *J* 1, 3 and 17, CH=C*H*H), 5.14–5.09 (1H, m, CH=CH*H*), 4.46–4.37 (3H, m, $C^{\alpha}H$(Pro_1)/$C^{\alpha}H$(Pro_2)/$C^{\alpha}H$(Ser(All))), 4.32 (1H, dd, *J* 7 and 9, $C^{\alpha}H$(Val_y)), 4.15–4.10 (1H, m, $C^{\alpha}H$(Val_x)), 3.93 (2H, m, *J* 1 and 5, CH_2CH=CH_2), 3.67–3.50 and 3.36–3.24 (6H, m, $C^{\delta}H_2$(Pro_1)/$C^{\delta}H_2$(Pro_2)/CH_2(Ser(All))), 3.61 (3H, s, OCH_3), 2.26–1.71 (10H, m, CH_2(Pro)/C*H*(CH_3)$_2$), 1.37 (9H, s, (CH_3)$_3$, rotamer 1), 1.30 (9H, s, (CH_3)$_3$, rotamer 2), 0.89–0.80 (12H, m, CH_3); δ_C (75 MHz; d_6-DMSO) 171.6, 171.5, 171.3, 171.1, 170.9, 170.4, 169.1, 169.1, 153.3, 152.9, 134.8, 116.5, 78.3, 78.1, 71.1, 69.3, 59.1, 57.4, 57.2, 56.9, 52.9, 51.4, 46.5, 46.4, 46.3, 46.3, 30.9, 30.6, 29.6, 29.5, 29.4, 28.7, 28.5, 28.1, 27.9, 24.4, 24.3, 23.6, 23.0; *m/z* (ESI) 674.3737 ([M + Na]$^+$; $C_{32}H_{53}N_5O_9Na$ requires 674.3741).

***N-tert*-Butoxycarbonyl *O*-allyl L-seryl α,α-dimethylglycyl L-valine methyl ester (12).** α,α-Dimethylglycyl L-valine methyl ester trifluoroacetate **7** (19.20 g, 58.13 mmol) was dissolved in DMF (100 mL). *N-tert*-Butoxycarbonyl *O*-allyl L-serine **3** (14.25 g, 58.12 mmol) and then *N*,*N*-diisopropylethylamine (7.51 g, 58.1 mmol) were added and the solution cooled to 0 °C (ice bath). HOBt hydrate (8.91 g, 58.2 mmol) and then EDC hydrochloride (12.26 g, 63.95 mmol) were added together with an additional 70 mL DMF. The reaction mixture was stirred for 22 hours before the solvent was evaporated. The residue was taken up in EtOAc (350 mL) and washed with 2 M aqueous H_2SO_4 (3 × 130 mL), 7.5% (w/w) aqueous K_2CO_3 (3 × 130 mL) and saturated brine (130 mL). The solution was dried with anhydrous $MgSO_4$ and the solvent evaporated affording an off-white solid. ^{1}H-NMR indicated 10–15% epimerization. The residue was recrystallized twice from ethyl acetate/hexane (4:1) to give the title compound as a stereopure off-white solid (20.15 g, 78% (sum of diastereomers)/13.49 g, 52%); δ_H (300 MHz; d_6-DMSO) 8.01 (1H, s, N*H*(Aib)), 7.29 (1H, d, *J* 8, N*H*(Val)), 6.89 (1H, d, *J* 7, N*H*(Ser(All))), 5.92–5.79 (1H, m, C*H*=CH_2), 5.29–5.22 (1H, m, CH=CH*H*), 5.15–5.11 (1H, m, CH=C*H*H), 4.18–4.12 (2H, m, $C^{\alpha}H$(Ser(All))/$C^{\alpha}H$(Val)), 3.97–3.94 (2H, m, CH_2CH=CH_2), 3.62 (3H, s, OCH_3), 3.57–3.46 (2H, m, CH_2), 2.08–1.93 (1H, m, C*H*(CH_3)$_2$), 1.38 (9H, s, (CH_3)$_3$), 1.37 (3H, s, CH_3(Aib)), 1.35 (3H, s, CH_3(Aib)), 0.83 (3H, d, *J* 7, CH_3(Val)), 0.82 (3H, d, *J* 7, CH_3(Val)); δ_C (75 MHz; d_6-DMSO) 173.8, 171.7, 169.5, 155.2, 134.8, 116.3, 78.2, 70.9, 69.4, 57.4, 56.1, 54.3, 51.5, 29.9, 28.0, 25.6, 23.8, 18.8, 18.1; *m/z* 466.2514 ([M + Na]$^+$; $C_{21}H_{37}N_3O_7Na$ requires 466.2529).

***N-tert*-Butoxycarbonyl *O*-benzyl L-seryl L-valyl L-valine methyl ester (13).** L-Valyl L-valine methyl ester trifluoroacetate **6** (11.62 g, 33.75 mmol) was dissolved in DMF (60 mL). *N-tert*-Butoxycarbonyl *O*-benzyl L-serine **14** (9.97 g, 33.8 mmol) was added together with 10 mL DMF. The solution was cooled to 0 °C (ice bath) before *N*,*N*-diisopropylethylamine (4.36 g, 33.7 mmol), HOBt hydrate (5.17 g, 33.8 mmol) and EDC hydrochloride (7.12 g, 37.1 mmol) were added together with an additional 30 mL DMF. The reaction mixture was stirred overnight and the solvent evaporated. The residue was taken up in 200 mL EtOAc and washed with 2 M aqueous H_2SO_4 (3 × 80 mL), 7.5% (w/w) aqueous K_2CO_3 (3 × 80 mL) and saturated brine (80 mL). The solution was dried with anhydrous $MgSO_4$ and the solvent evaporated affording a white solid (16.22 g, 95%); δ_H (300 MHz; d_6-DMSO) 8.20 (1H, d, *J* 8, N*H*(Val_x)), 7.65 (1H, d, *J* 9, N*H*(Val_y)), 7.35–7.23 (5H, m, *Ph*), 7.08 (1H, d, *J* 8, N*H*(Ser(Bn)), 4.46 (2H, s, CH_2Ph), 4.35 (1H, dd, *J* 7 and 9, $C^{\alpha}H$(Val_y)), 4.25 (1H, m, $C^{\alpha}H$(Ser(Bn))), 4.12 (1H, dd, *J* 6 and 8, $C^{\alpha}H$(Val_x)), 3.63–3.54 (2H, m, CH_2), 3.60 (3H, s, OCH_3), 2.07–1.89 (2H, m, C*H*(CH_3)$_2$), 1.38 (9H, s, (CH_3)$_3$), 0.88–0.80 (12H, m, CH_3); δ_C (75 MHz; d_6-DMSO) 171.6, 171.0, 169.4, 155.1, 138.0, 128.0, 127.3, 127.3, 78.2, 71.9, 69.8, 57.4, 56.8, 54.5, 51.4, 31.1, 29.5, 28.0, 18.9, 18.7, 18.2, 17.7; *m/z* (ESI) 530.2824 ([M + Na]$^+$; $C_{26}H_{41}N_3O_7Na$ requires 530.2842).

***N-tert*-Butoxycarbonyl *O*-allyl L-seryl L-valyl L-valine (15).** *N-tert*-Butoxycarbonyl *O*-allyl L-seryl L-valyl L-valine methyl ester **8** (10.21 g, 22.31 mmol) was dissolved in THF (170 mL). The solution was cooled to 0 °C (ice bath). LiOH monohydrate (1.03 g, 24.6 mmol) was dissolved in de-ionized water (85 mL) and the solution cooled to 0 °C. The solution was then added dropwise to the solution of **8** over 20 min. The reaction mixture was stirred for an additional 2 h 40 min at 0 °C. Solid $NaHCO_3$ (3.75 g, 44.6 mmol) was added and the mixture stirred for 5 min before the THF was evaporated. The solution was diluted with water (170 mL), washed with Et_2O (2 × 170 mL), acidified to pH 2 by addition of 2 M aqueous H_2SO_4 and extracted with EtOAc (3 × 250 mL). The combined organic fractions were dried with anhydrous $MgSO_4$ and the solvent evaporated affording the title compound as a white solid (7.00 g, 71%); δ_H (300 MHz; d_6-DMSO) 12.53 (1H, br s, COO*H*), 8.00 (1H, d, *J* 8, N*H* (Val_x)), 7.62 (1H, d, *J* 9, N*H*(Val_y)), 7.02 (1H, d, *J* 8, N*H*(Ser(All))), 5.84 (1H, ddt, *J* 5, 10 and 17, C*H*=CH_2), 5.23 (1H, ddt, *J* 1, 3 and 17, CH=C*H*H)), 5.12 (1H, ddt, *J* 1, 3 and 10, CH=CH*H*), 4.34 (1H, dd, *J* 7 and 9, $C^{\alpha}H$(Val_y)), 4.21–4.15 (1H, m, $C^{\alpha}H$(Ser(All))), 4.10 (1H, dd, *J* 6 and 8, $C^{\alpha}H$(Val_x)), 3.92 (2H, dt, *J* 1 and 5, CH_2CH=CH_2), 3.59–3.45 (2H, m, CH_2), 2.06–1.91 (2H, m, C*H*(CH_3)$_2$), 1.38 (9H, s, (CH_3)$_3$), 0.90–0.80 (12H, m, CH_3); δ_C (75 MHz; d_6-DMSO) 172.6, 170.9, 169.5, 155.1, 134.9, 116.3, 78.2, 71.0, 69.5, 57.2, 56.9, 54.6, 31.1, 29.6, 28.1, 19.0, 19.0, 17.9, 17.7; *m/z* (ESI) 466.2518 ([M + Na]$^+$; $C_{21}H_{37}N_3O_7Na$ requires 466.2529).

***N-tert*-Butoxycarbonyl *O*-allyl L-seryl α,α-dimethylglycyl L-valine (16).** *N-tert*-Butoxycarbonyl *O*-allyl L-seryl α,α-dimethylglycyl L-valine methyl ester **12** (8.56 g, 19.3 mmol) was dissolved in THF (150 mL). The solution was cooled to 0 °C (ice bath). LiOH monohydrate (0.891 g, 21.2 mmol) was dissolved in de-ionized water (75 mL) and the solution cooled to 0 °C. The solution was added dropwise to the solution of **12** over 20 min. The reaction mixture was stirred for an additional 2 h 40 min before solid $NaHCO_3$ (3.24 g, 38.6 mmol) was added. The mixture was stirred for 5 min and the bulk of THF evaporated. The solution was diluted with water (150 mL), washed with Et_2O (2 × 150 mL), acidified to pH 2 by addition of 2 M aqueous H_2SO_4 and extracted with EtOAc (3 × 300 mL). The combined organic fractions were dried with anhydrous $MgSO_4$ and the solvent evaporated affording the title compound as a white solid (7.30 g, 88%); δ_H (300 MHz; d_6-DMSO) 12.62 (1H, br s, COO*H*), 8.01 (1H, s, N*H*(Aib)), 7.12 (1H, d, *J* 9, N*H*(Val)), 6.85 (1H, d, *J* 8, N*H*(Ser(All))), 5.92–5.79 (1H, m, C*H*=CH_2), 5.29–5.21 (1H, m, CH=C*H*H), 5.15–5.10 (1H, m, CH=CH*H*), 4.19–4.10 (2H, m, $C^{\alpha}H$(Ser(All))/$C^{\alpha}H$(Val)), 3.94 (2H, dt, *J* 1 and 5, CH_2CH=CH_2), 3.57–3.45 (2H, m, CH_2), 2.08–1.97 (1H, m, C*H*$(CH_3)_2$), 1.38 (12H, s, CH_3(Aib)/$(CH_3)_3$), 1.35 (3H, s, CH_3(Aib)), 0.84 (3H, d, *J* 7, CH_3(Val)), 0.81 (3H, d, *J* 7, CH_3(Val)); δ_C (75 MHz; d_6-DMSO): 173.6, 172.7, 169.5, 155.1, 134.9, 116.3, 78.2, 70.9, 69.5, 57.0, 56.1, 54.4, 30.1, 28.1, 25.7, 23.8, 19.0, 17.8; *m/z* (ESI) 452.2357 ([M + Na]$^+$; $C_{20}H_{35}N_3O_7Na$ requires 452.2372).

***N-tert*-Butoxycarbonyl *O*-benzyl L-seryl L-valyl L-valine (17).** *N-tert*-Butoxycarbonyl *O*-benzyl L-seryl L-valyl L-valine methyl ester **13** (14.60 g, 28.76 mmol) was dissolved in THF (220 mL) and the solution cooled to 0 °C (ice bath). LiOH monohydrate (1.33 g, 31.7 mmol) was dissolved in de-ionized water (110 mL). The solution was cooled to 0 °C (ice bath) and added dropwise to the solution of **13** over 25 min. The reaction mixture was stirred for an additional 2 h 35 min before solid $NaHCO_3$ (4.83 g, 57.5 mmol) was added and the bulk of THF evaporated. The solution was diluted with water (220 mL) and washed with Et_2O (2 × 220 mL). The solution was then acidified to pH 2 by addition of 2 M aqueous H_2SO_4 and extracted with EtOAc (3 × 350 mL). The combined organic fractions were dried with anhydrous $MgSO_4$ and the solvent evaporated affording a slightly yellowish liquid. The residue was redissolved in EtOAc and precipitated by addition of hexane. The solvent was decanted off and the residue dried affording the title compound as an off-white solid (5.95 g, 42%); δ_H (200 MHz; d_6-DMSO) 12.52 (1H, br s, COO*H*), 8.03 (1H, d, *J* 8, N*H*(Val_x)), 7.65 (1H, d, *J* 9, N*H*(Val_y)), 7.36–7.23 (5H, m, *Ph*), 7.10 (1H, d, *J* 8, N*H*(Ser(Bn)), 4.46 (2H, s, CH_2Ph), 4.36 (1H, dd, *J* 7 and 9, $C^{\alpha}H(Val_y)$), 4.28–4.22 (1H, m, $C^{\alpha}H$(Ser(Bn))), 4.10 (1H, dd, *J* 6 and 8, $C^{\alpha}H(Val_x)$), 3.64–3.54 (2H, m, CH_2), 2.08–1.91 (2H, m, C*H*$(CH_3)_2$), 1.38 (9H, s, $(CH_3)_2$), 0.88–0.80 (12H, m, CH_3); δ_C (75 MHz; d_6-DMSO) 172.6, 170.9, 169.4, 155.1, 138.1, 128.0, 127.4, 127.3, 78.3, 71.9, 69.8, 57.2, 56.8, 54.6, 31.1, 29.5, 28.1, 19.0, 18.9, 18.0, 17.7; *m/z* (ESI) 516.2663 ([M + Na]$^+$; $C_{25}H_{39}N_3O_7Na$ requires 516.2685).

***N-tert*-Butoxycarbonyl *O*-allyl L-seryl glycine methyl ester (18).** Purified *N-tert*-butoxycarbonyl *O*-allyl L-serine **3** (6.94 g, 28.3 mmol) was dissolved in DMF (30 mL) and the solution cooled to 0 °C (ice bath). Glycine methyl ester hydrochloride (3.55 g, 28.3 mmol) was suspended in DMF (10 mL) and *N*,*N*-diisopropylethylamine (3.66 g, 28.3 mmol) added. The suspension was then added in one portion to the solution of **3** together with an additional 5 mL DMF. HOBt hydrate (4.33 g, 28.3 mmol) was added. Finally, EDC hydrochloride (5.97 g, 31.1 mmol) was added together with an additional 10 mL DMF. The reaction mixture was stirred at 0 °C for 1 hour and then at room temperature for 23 hours before the solvent was evaporated. The residue was taken up in EtOAc (150 mL) and washed with 1 M aqueous H_2SO_4 (3 × 100 mL), 7.5% (w/w) aqueous K_2CO_3 (3 × 100 mL) and finally saturated brine (100 mL). The solution was dried with anhydrous $MgSO_4$ and the solvent evaporated affording a clear and slightly greenish/yellowish liquid (7.49 g, 84%); δ_H (200 MHz; d_6-DMSO) 8.31 (1H, t, *J* 6, N*H*(Gly)), 6.85 (1H, d, *J* 8, N*H*(Ser(All))), 5.86 (1H, ddt, *J* 5, 11 and 17, C*H*=CH_2), 5.29–5.11 (2H, m, CH=CH_2), 4.22 (1H, ddd, *J* 8 and 12, $C^{\alpha}H$(Ser(All))), 3.94 (2H, dt, *J* 1 and 5, CH_2CH=CH_2), 3.86 (1H, d, *J* 6, $C^{\alpha}H$H(Gly)), 3.83 (1H, d, *J* 6, C^{α}H*H*(Gly)), 3.62 (3H, s, OCH_3), 3.56–3.44 (2H, m, CH_2), 1.38 (9H, s, $(CH_3)_3$); δ_C (50 MHz; d_6-DMSO) 170.3, 170.0, 155.1, 134.9, 116.3, 78.2, 70.9, 69.6, 54.1, 51.6, 40.5, 28.1; *m/z* (ESI) 339.1541 ([M + Na]$^+$; $C_{14}H_{24}N_2O_6Na$ requires 339.1532).

***O*-Allyl L-seryl glycine methyl ester trifluoroacetate (20).** *N-tert*-Butoxycarbonyl *O*-allyl L-seryl glycine methyl ester **18** (5.86 g, 18.3 mmol) was dissolved in CH_2Cl_2 (30 mL) and TFA (30 mL) added at room temperature. The reaction mixture was stirred for 2 hours before the solvent and bulk of excess TFA were evaporated affording a brownish liquid (7.50 g). The residue was washed with Et_2O (2 × 25 mL), redissolved in CH_2Cl_2 (25 mL), the solvents evaporated and the residue dried under reduced pressure affording a brownish, viscous liquid (6.01 g, 98%); δ_H (300 MHz; d_6-DMSO) 9.02 (1H, t, *J* 6, N*H*), 8.33 (3H, br s, NH_3^+), 5.87 (1H, ddt, *J* 5, 10 and 17, C*H*=CH_2), 5.29 (1H, ddt, *J* 1, 2 and 17, CH=C*H*H), 5.21–5.14 (1H, m, CH=CH*H*), 4.10 (1H, m, $C^{\alpha}H$(Ser(All))), 4.00 (2H, ddd, *J* 1, 1 and 5, CH_2CH=CH_2), 3.95 (2H, d, *J* 6, $C^{\alpha}H_2$(Gly)), 3.78–3.67 (2H, m, CH_2), 3.64 (3H, s, OCH_3); δ_C (75 MHz; d_6-DMSO) 169.7, 167.0, 158.4 (q, J_{CF} 32), 134.4, 116.7 (q, J_{CF} 296), 71.4, 68.0, 54.9, 52.3, 51.8, 40.7; *m/z* (ESI) 217.1194 (M$^+$; $C_9H_{17}N_2O_4$ requires 217.1188).

***N-tert*-Butoxycarbonyl *O*-allyl L-seryl L-valyl L-valyl *O*-allyl L-seryl glycine methyl ester (22).** *O*-Allyl L-seryl methyl ester trifluoroacetate **20** (7.03 g, 21.3 mmol), *N-tert*-butoxycarbonyl *O*-allyl L-seryl L-valyl L-valine **15** (9.45 g, 21.3 mmol) and *N*,*N*-diisopropylethylamine (2.75 g, 21.3 mmol) were dissolved in CH_2Cl_2 (100 mL) and the solution cooled to 0 °C (ice bath). HOBt hydrate (3.26 g, 21.3 mmol) and then EDC hydrochloride (4.49 g, 23.4 mmol) were added in small portions. The reaction mixture was stirred for 1 h 30 min at 0 °C after which the ice bath was removed and the mixture stirred for 14 h 30 min. The volume was then increased by addition of CH_2Cl_2 (200 mL) and the mixture stirred for another 7 hours before the volume was increased to 400 mL. The suspension was washed with 2 M aqueous H_2SO_4 (150 + 300 + 450 mL), 7.5% (w/w) aqueous K_2CO_3 (150 + 300 + 450 mL), saturated brine (400 mL) and water (400 + 600 mL). The suspension was then diluted with CH_2Cl_2 (1.5 L) and dried with 3 Å molecular sieves resulting in a slightly turbid solution. The solvent was evaporated affording a slightly yellowish solid, which was recrystallised from EtOH affording the title compound as a white solid (12.12 g, 89%); δ_H (300 MHz; d_6-DMSO) 8.36 (1H, t, *J* 6,

N*H*(Gly)), 7.98 (1H, d, *J* 8, N*H*(Ser(All)$_2$)), 7.90 (1H, d, *J* 9, N*H*(Val$_x$)), 7.68 (1H, d, *J* 9, N*H*(Val$_y$)), 7.02 (1H, d, *J* 8, N*H*(Ser(All)$_1$)), 5.90–5.77 (2H, m, C*H*=CH$_2$), 5.27–5.10 (4H, m, CH=C*H*$_2$), 4.55–4.49 (1H, m, C$^\alpha$*H*(Ser(All)$_2$)), 4.30–4.15 (3H, m, C$^\alpha$*H*(Val$_x$)/C$^\alpha$*H*(Val$_y$)/C$^\alpha$*H*(Ser(All)$_1$)), 3.95–3.91 (4H, m, C*H*$_2$CH=CH$_2$), 3.86–3.84 (2H, m, C$^\alpha$*H*$_2$(Gly)), 3.61 (3H, s, OC*H*$_3$), 3.56–3.46 (4H, m, C*H*$_2$), 2.02–1.89 (2H, m, C*H*(CH$_3$)$_2$), 1.38 (9H, s, (C*H*$_3$)$_3$), 0.84–0.78 (12H, m, C*H*$_3$); δ_C (75 MHz; d$_6$-DMSO) 170.6, 169.9, 169.7, 169.5, 155.1, 134.9, 134.8, 116.5, 116.3, 79.8, 79.7, 78.2, 71.0, 69.5, 69.4, 57.4, 57.2, 57.2, 54.6, 54.5, 54.5, 52.3, 51.6, 40.5, 30.8, 30.3, 28.0, 19.0, 17.9, 17.8; *m*/*z* (ESI) 664.3526 ([M + Na]$^+$; $C_{30}H_{51}N_5O_{10}Na$ requires 664.3533).

***N*-*tert*-Butoxycarbonyl *O*-allyl L-seryl α,α-dimethylglycyl L-valyl *O*-allyl L-seryl glycine methyl ester (23).** *O*-Allyl L-seryl glycine methyl ester trifluoroacetate **20** (4.42 g, 13.4 mmol) was dissolved in CH_2Cl_2 (40 mL) and *N*-*tert*-butoxycarbonyl *O*-allyl L-seryl α,α-dimethylglycyl L-valine **16** (5.75 g, 13.4 mmol) added. *N*,*N*-diisopropylethylamine (1.73 g, 13.4 mmol) and HOBt hydrate (2.05 g, 13.4 mmol) were added and the solution cooled to 0 °C. EDC hydrochloride (2.82 g, 14.7 mmol) was added in small portions together with 10 mL CH_2Cl_2. The reaction mixture was stirred for 1 h 30 min at 0 °C after which the ice bath was removed and the mixture stirred for an additional 26 hours. The solution was diluted by addition of CH_2Cl_2 (60 mL) and washed with 2 M aqueous H_2SO_4 (3 × 90 mL), 7.5% (w/w) aqueous K_2CO_3 (3 × 90 mL) and saturated brine (90 mL). The volume was increased by addition of CH_2Cl_2, the solution dried with anhydrous $MgSO_4$ and the solvent evaporated affording a slightly orange solid (6.14 g, 73%); δ_H (300 MHz; d$_6$-DMSO) 8.25 (1H, t, *J* 6, N*H*(Gly)), 8.14 (1H, s, N*H*(Aib)), 7.88 (1H, d, *J* 8, N*H*(Ser(All))), 7.14 (1H, d, *J* 8, N*H*(Val)), 6.84 (1H, d, *J* 7, N*H* (Ser(All)$_1$)), 5.92–5.74 (2H, m, C*H*=CH$_2$), 5.28–5.11 (4H, m, CH=C*H*$_2$), 4.50 (1H, m, C$^\alpha$*H*(Ser(All)$_2$)), 4.17–4.09 (2H, m, C$^\alpha$*H*(Ser(All)$_1$)/C$^\alpha$*H*(Val)), 3.95 (4H, m, C*H*$_2$CH=CH$_2$), 3.85 (2H, d, *J* 6, C$^\alpha$*H*$_2$(Gly)), 3.61 (3H, s, OC*H*$_3$), 3.58–3.46 (4H, m, C*H*$_2$), 2.06–1.95 (1H, m, C*H*(CH$_3$)$_2$), 1.38 (9H, s, (C*H*$_3$)$_3$), 1.35 (3H, s, C*H*$_3$(Aib)), 1.34 (3H, s, C*H*$_3$(Aib)), 0.83 (3H, d, *J* 7, C*H*$_3$(Val)), 0.78 (3H, d, *J* 7, C*H*$_3$(Val)); δ_C (75 MHz; d$_6$-DMSO) 173.9, 170.7, 169.9, 169.8, 169.7, 155.3, 134.8, 116.4, 116.3, 78.4, 71.0, 69.4, 58.2, 56.2, 54.8, 54.5, 52.5, 51.6, 40.6, 30.0, 28.0, 24.9, 24.7, 19.0, 17.8; *m*/*z* 650.3366 ([M + Na]$^+$; $C_{29}H_{49}N_5O_{10}Na$ requires 650.3377).

***N*-*tert*-Butoxycarbonyl *O*-benzyl L-seryl L-valyl L-valyl glycyl glycine methyl ester (24).** *N*-*tert*-Butoxycarbonyl *O*-benzyl L-seryl L-valyl L-valine **17** (2.20 g, 4.46 mmol) and glycyl glycine methyl ester trifluoroacetate **21** (1.16 g, 4.44 mmol) were dissolved in CH_2Cl_2 (15 mL). *N*,*N*-Diisopropylethylamine (0.57 g, 4.4 mmol) and HOBt hydrate (0.68 g, 4.4 mmol) were added and the solution cooled to 0 °C. EDC hydrochloride (0.94 g, 4.9 mmol) was added slowly together with 5 mL CH_2Cl_2. The reaction mixture was stirred for 1 h 30 min at 0 °C after which the ice bath was removed. The mixture was stirred for an additional 23 hours before the volume was increased by addition of CH_2Cl_2 (20 mL). The solution was washed with 2 M aqueous H_2SO_4 (3 × 30 mL), 7.5% (w/w) aqueous K_2CO_3 (3 × 30 mL) and saturated brine (30 mL). The volume of the solution was increased, the solution dried with anhydrous $MgSO_4$ and the solvent evaporated affording an off-white solid (2.14 g, 78%); δ_H (300 MHz; d$_6$-DMSO) 8.25–8.20 (2H, m, N*H*(Gly$_1$)/N*H*(Gly$_2$)), 7.93 (1H, d, *J* 8, N*H*(Val$_x$)), 7.70 (1H, d, *J* 9, N*H*(Val$_y$)), 7.35–7.25 (5H, m, *Ph*), 7.06 (1H, d, *J* 8, N*H*(Ser(Bn))), 4.47 (2H, s, C*H*$_2$Ph), 4.32–4.23 (2H, m, C$^\alpha$*H*(Ser(Bn))/C$^\alpha$*H*(Val)), 4.11 (1H, dd, *J* 8 and 8, C$^\alpha$*H*(Val)), 3.89 (1H, dd, *J* 6 and 17, C$^\alpha$*H*H(Gly)), 3.82 (1H, dd, *J* 6 and 17, C$^\alpha$H*H*(Gly)), 3.75 (2H, m, C$^\alpha$*H*$_2$(Gly)), 3.62 (3H, s, OC*H*$_3$), 3.60–3.53 (2H, m, C*H*$_2$), 2.01–1.88 (2H, m, C*H*(CH$_3$)$_2$), 1.38 (9H, s, (C*H*$_3$)$_3$), 0.85–0.78 (12H, m, C*H*$_3$); δ_C (75 MHz; d$_6$-DMSO) 171.1, 170.9, 170.1, 169.6, 169.1, 155.2, 138.1, 128.0, 127.4, 127.3, 78.2, 71.9, 69.8, 58.1, 57.2, 54.5, 51.6, 41.6, 40.5, 30.8, 30.1, 28.1, 19.1, 18.2, 17.8; *m*/*z* (ESI) 644.3268 ([M + Na]$^+$; $C_{30}H_{47}N_5O_9Na$ requires 644.3271).

Methyl 2-((3S,6S,9S,12S,E)-12-(*tert*-butoxycarbonylamino)-6,9-diisopropyl-5,8,11-trioxo-1,14-dioxa-4,7,10-triazacyclooctadec-16-ene-carboxamido)-acetate (25). *N*-*tert*-Butoxycarbonyl *O*-allyl L-seryl L-valyl L-valyl *O*-allyl L-seryl glycine methyl ester **22** (0.642 g, 1.00 mmol) was dissolved in dry, degassed CH_2Cl_2 (225 mL). Grubbs' 2nd generation catalyst (0.085 g, 0.10 mmol) dissolved in dry, degassed CH_2Cl_2 (25 mL) was added by syringe and the reaction mixture stirred under argon atmosphere for 2 h 45 min. A second portion of Grubbs' 2nd generation catalyst (0.085 g, 0.10 mmol) dissolved in CH_2Cl_2 (20 mL) was then added and the reaction mixture stirred for an additional 4 h 45 min. Ethyl vinyl ether (1.5 mL) was added to quench the reaction/remaining catalyst. After stirring for 25 min the solvent was evaporated affording a dark residue. The residue was redissolved in a minimal amount of CH_2Cl_2 and purified by flash column chromatography (eluent: CH_2Cl_2/acetone (2:1)) affording the title compound as an off-white solid (0.256 g, 42%); δ_H (300 MHz; $CDCl_3$) 7.23–7.20 (2H, m, N*H*(Val$_x$)/N*H*(Gly)), 7.11 (1H, d, *J* 8, N*H*(Ser(All)$_2$)), 6.75 (1H, d, *J* 7, N*H*(Val$_y$)), 5.92–5.80 (2H, m, C*H*=C*H*), 5.35 (1H, d, *J* 6, N*H*(Ser(All)$_1$)), 4.71–4.65 (1H, m, C$^\alpha$*H*(Ser(All)$_2$)), 4.37 (1H, dd, *J* 6 and 12, C$^\alpha$*H*(Ser(All)$_1$)), 4.18–4.11 (3H, m, CH*H*CH=CH/C$^\alpha$*H*(Val$_x$)/C$^\alpha$*H*(Val$_y$)), 4.08 (1H, d, *J* 6, C$^\alpha$*H*H(Gly)), 4.02–3.91 (5H, m, C*H*H(Ser(All)$_2$)/C*H*$_2$CH=CH/C*H*HCH=CH/C$^\alpha$H*H*(Gly)), 3.77–3.69 (2H, m, C*H*$_2$(Ser(All)$_1$)), 3.73 (3H, s, OC*H*$_3$), 3.63 (1H, dd, *J* 5 and 9, CH*H*(Ser(All)$_2$)), 2.35–2.21 (2H, m, C*H*(CH$_3$)$_2$), 1.46 (9H, s, (C*H*$_3$)$_3$), 1.02–0.90 (12H, m, C*H*$_3$); δ_C (75 MHz; $CDCl_3$) 172.0, 171.5, 171.3, 170.0, 156.3, 130.7, 128.7, 80.8, 77.2, 71.2, 70.0, 68.9, 67.7, 60.5, 60.4, 54.3, 53.3, 52.2, 41.3, 29.9, 29.3, 28.2, 19.5, 19.5, 18.0, 17.3; *m*/*z* (ESI) 636.3209 ([M + Na]$^+$; $C_{28}H_{47}N_5O_{10}Na$ requires 636.3220).

Methyl 2-((3S,6S,12S,E)-12-(*tert*-butoxycarbonylamino)-6-isopropyl-9,9-dimethyl-5,8,11-trioxo-1,14-dioxa-4,7,10-triazacyclooctadec-16-enecarboxamido)acetate (26). *N*-*tert*-Butoxycarbonyl *O*-allyl L-seryl α,α-dimethylglycyl L-valyl *O*-allyl L-seryl glycine methyl ester **23** (0.571 g, 0.910 mmol) was dissolved in dry CH_2Cl_2 (200 mL) and the solution degassed. Grubbs' 2nd generation catalyst (0.077 g, 0.091 mmol) was dissolved in dry, degassed CH_2Cl_2 (25 mL) and added to the solution of **23** by syringe. The reaction mixture was stirred under argon atmosphere for 3 hours. A new portion of Grubbs' 2nd generation catalyst (0.039 g, 0.046 mmol) dissolved in dry, degassed CH_2Cl_2 (10 mL) was then added. After stirring for 3 hours ethyl vinyl ether (1.0 mL) was added to quench the reaction/remaining catalyst. The reaction mixture was stirred for an additional 30 min before the solvent was evaporated. The residue was purified

by flash column chromatography (eluent: CH_2Cl_2/acetone (2:1)) affording a glassy/transparent solid (0.286 g, 52%); δ_H (300 MHz; $CDCl_3$) 7.56–7.40 (2H, m, N*H*(Ser(All)$_2$)/N*H*(Gly)), 7.13 (1H, s, N*H*(Aib)), 6.93 (1H, d, *J* 5, N*H*(Val)), 5.81–5.65 (2H, m, C*H*=C*H*), 5.49 (1H, d, *J* 5, N*H*(Ser(All)$_1$)), 4.79–4.73 (1H, m, C$^\alpha$*H*(Ser(All)$_2$)), 4.25–4.19 (1H, m, C$^\alpha$*H*(Ser$_1$)), 4.11–4.00 (4H, m, C$^\alpha$*H*(Val)/C$^\alpha$*H*H(Gly)/C*H*$_2$CH=CH), 3.89–3.74 (6H, m, C*H*$_2$CH=CH/C$^\alpha$H*H*(Gly)/C*H*$_2$(Ser(All)$_2$))/C*H*H(Ser$_1$)), 3.66 (3H, s, OC*H*$_3$), 3.59–3.49 (1H, m, CH*H*(Ser$_1$)), 2.42–2.25 (1H, m, C*H*(CH$_3$)$_2$), 1.47–1.41 (15H, m, C*H*$_3$(Aib)/(C*H*$_3$)$_3$), 1.00–0.92 (6H, m, C*H*$_3$(Val)); δ_C (75 MHz; $CDCl_3$) 175.6, 171.2, 171.0, 170.1, 169.9, 156.0, 131.0, 127.7, 80.7, 70.5, 69.5, 69.2, 67.0, 60.6, 57.3, 55.0, 53.8, 51.9, 41.1, 29.0, 28.1, 26.3, 23.7, 19.3, 17.4; *m*/*z* (ESI) 622.3054 ([M + Na]$^+$; $C_{27}H_{45}N_5O_{10}Na$ requires 622.3064).

(3S,6S,9S,12S,E)-6,9-Diisopropyl-3-(2-methoxy-2-oxoethylcarbamoyl)-5,8,11-trioxo-1,14-dioxa-4,7,10-triazacyclooctadec-16-en12-aminium 2,2,2-trifluoroacetate (27). Cyclic pentapeptide **25** (0.403 g, 0.657 mmol) was dissolved in a 50% (v/v) solution of TFA in CH_2Cl_2 (10 mL) at room temperature. The reaction mixture was stirred for 2 hours before the solvent and excess TFA were evaporated affording an off-white solid. Dichloromethane (10 mL) was added and evaporated and the residue washed with Et_2O (2 × 5 mL) to give the title compound as a slightly off-white solid (0.392 g, 95%); δ_H (300 MHz; d_6-DMSO) 8.76 (1H, d, *J* 9, N*H*(Val$_x$)), 8.58 (1H, t, *J* 6, N*H*(Gly)), 8.37–8.22 (4H, m, N*H*(Ser(All)$_2$)/N*H*$_3^+$), 7.31 (1H, d, *J* 8, N*H*(Val$_y$)), 5.80–5.67 (2H, m, C*H*=C*H*), 4.84–4.77 (1H, m, C$^\alpha$*H*(Ser(All)$_2$)), 4.32 (1H, dd, *J* 6 and 8, C$^\alpha$*H*(Val$_y$)), 4.13–3.93 (6H, m, C*H*$_2$CH=CH$_2$/C$^\alpha$*H*(Val$_x$)/C$^\alpha$*H*(Ser(All)$_1$)), 3.90–3.87 (2H, m, C$^\alpha$*H*$_2$(Gly)), 3.70 (1H, dd, *J* 4 and 10, CH*H*(Ser(All)$_1$)), 3.62 (3H, s, OC*H*$_3$), 3.55–3.42 (3H, m, C*H*$_2$(Ser(All)$_2$)/C*H*H(Ser(All)$_1$)), 2.10–1.89 (2H, m, C*H*(CH$_3$)$_2$), 0.91–0.83 (12H, m, C*H*$_3$); δ_C (75 MHz; d_6-DMSO) 169.9, 169.8, 169.6, 169.5, 166.3, 130.6, 128.6, 70.9, 68.9, 68.7, 67.5, 59.8, 56.9, 52.6, 51.6, 50.8, 40.5, 31.4, 30.3, 19.2, 18.7, 18.3, 18.2; *m*/*z* (ESI) 514.2868 (M$^+$; $C_{23}H_{40}N_5O_8$ requires 514.2876).

(3S,6S,9S,12S,E)-6-Isopropyl-3-(2-methoxy-2-oxoethylcarbamoyl)-9,9-dimethyl-5,8,11-trioxo-1,14-dioxa-4,7,10-triazacyclooctadec-16-en-12-aminium 2,2,2-trifluoroacetate (28). Cyclic pentapeptide **26** (0.426 g, 0.710 mmol) was dissolved in a 50% (v/v) solution of TFA in CH_2Cl_2 (10 mL). The reaction mixture was stirred for 1 hour at room temperature before the solvent and bulk of excess TFA were evaporated. Et_2O (10 mL) was added to the liquid residue, which resulted in precipitation of a white solid. After decanting off the Et_2O the residue was washed with additional portions of Et_2O (2 × 10 mL) and dried affording a white solid (0.309 g, 71%); δ_H (300 MHz; d_6-DMSO) 8.80 (1H, s, N*H*(Aib)), 8.49 (1H, t, *J* 6, N*H*(Gly)), 8.28 (3H, br s, N*H*$_3^+$), 8.13 (1H, d, *J* 8, N*H*(Ser(All)$_2$)), 7.11 (1H, d, *J* 7, N*H*(Val)), 5.84–5.72 (2H, m, C*H*=C*H*), 4.68–4.61 (1H, m, C$^\alpha$*H*(Ser(All)$_2$)), 4.24–4.03 (4H, m, C$^\alpha$*H*(Val)/C*H*$_2$CH=CH/CH*H*CH=CH), 3.96–3.91 (2H, m, C$^\alpha$*H*(Ser(All)$_1$)/C*H*HCH=CH), 3.88 (2H, dd, *J* 6 and 6, C$^\alpha$*H*$_2$(Gly)), 3.73 (1H, dd, *J* 4 and 10, C*H*H(Ser(All)$_1$)), 3.62 (3H, s, OC*H*$_3$), 3.54–3.39 (3H, m, C*H*$_2$(Ser(All)$_2$)/CH*H*(Ser(All)$_1$)), 2.04–1.93 (1H, m, C*H*(CH$_3$)$_2$), 1.43 (3H, s, C*H*$_3$(Aib), 1.38 (3H, s, C*H*$_3$(Aib)), 0.83 (3H, s, C*H*$_3$(Val)), 0.81 (3H, s, C*H*$_3$(Val)); δ_C (75 MHz; d_6-DMSO) 172.8, 170.1, 169.8, 169.7, 165.8, 131.0, 129.9, 70.4, 68.9, 67.8, 66.4, 57.4, 56.5, 53.6, 52.5, 51.6, 40.5, 30.8, 26.7, 22.7, 18.8, 18.1; *m*/*z* (ESI) 500.2713 (M$^+$; $C_{22}H_{38}N_5O_8$ requires 500.2720).

(S)-*tert*-Butyl 2-((S)-2-((3S,6S,9S,12S,E)-6,9-diisopropyl-3-(2-methoxy-2-oxoethylcarbamoyl)-5,8,11-trioxo-1,14-dioxa-4,7,10-triazacyclooctadec-16-en-12-ylcarbamoyl)pyrrolidine-1-carbonyl) pyrrolidine-1-carboxylate (29). Trifluoroacetate **27** (0.314 g, 0.500 mmol), *N*-*tert*-butoxycarbonyl L-prolyl L-proline **2** (0.157 g, 0.503 mmol) and HOBt hydrate (0.077 g, 0.50 mmol) were dissolved in CH_2Cl_2 (5 mL). *N*,*N*-Diisopropylethylamine (0.064 g, 0.50 mmol) dissolved in CH_2Cl_2 (1 mL) was added and the mixture stirred for 5 min. EDC hydrochloride (0.106 g, 0.553 mmol) was added in portions together with CH_2Cl_2 (4 mL). The reaction mixture was stirred for 1 hour at room temperature before CH_2Cl_2 (5 mL) was added. Stirring was continued for 21 hours after which the volume was increased to 50 mL by addition of CH_2Cl_2. The solution was washed with 1 M aqueous H_2SO_4 (3 × 20 mL), 7.5% (w/w) K_2CO_3 solution (3 × 20 mL) and saturated brine (20 mL). The solution was dried with anhydrous $MgSO_4$ and the solvent evaporated affording the title compound as an off-white solid (0.316 g, 78%); δ_H (300 MHz; CD_2Cl_2) 7.93 (1H, d, *J* 7, N*H*(Ser(All)$_1$)), 7.77 (1H, d, *J* 5, N*H*(Val$_1$)), 7.36–7.31 (2H, m, N*H*(Ser(All)$_2$)/N*H*(Gly)), 6.80 (1H, d, *J* 6, N*H*(Val$_2$)), 5.93–5.73 (2H, m, C*H*=C*H*), 4.73–4.66 (1H, m, C$^\alpha$*H*(Ser(All)$_1$)), 4.64–4.58 (1H, m, C$^\alpha$*H*(Ser(All)$_2$)), 4.40 (1H, dd, *J* 8 and 8, C$^\alpha$*H*(Pro$_2$), 4.33–4.27 (1H, m, C$^\alpha$*H*(Pro$_1$), 4.20–3.83 (11H, m, C$^\alpha$*H*(Val$_1$)/C$^\alpha$*H*(Val$_2$)/C$^\alpha$*H*$_2$(Gly)/C*H*$_2$(Ser(All)$_1$)/CH*H*(Ser(All)$_2$)/C*H*$_2$CH=CH), 3.71–3.58 (3H, m, C*H*H(Ser(All)$_2$)/C$^\delta$*H*$_2$(Pro)), 3.69 (3H, s, OC*H*$_3$), 3.46–3.29 (2H, m, C$^\delta$*H*$_2$(Pro)), 2.45–2.19 (4H, m, C*H*$_2$(Pro)), 2.11–1.77 (6H, m, C*H*(CH$_3$)$_2$/C*H*$_2$(Pro)), 1.47 (9H, s, (C*H*$_3$)$_3$), 1.07–0.96 (12H, m, C*H*$_3$); δ_C (75 MHz; CD_2Cl_2) 173.9, 173.4, 172.5, 172.3, 172.0, 170.9, 170.5, 155.5, 132.8, 130.8, 81.6, 71.2, 70.7, 69.7, 67.3, 63.5, 63.1, 63.0, 61.2, 55.6, 54.6, 52.4, 47.7, 47.4, 41.8, 29.6, 29.5, 29.4, 29.4, 28.6, 26.7, 25.2, 19.8, 19.3, 19.0, 17.8; *m*/*z* (ESI) 830.4269 ([M + Na]$^+$; $C_{38}H_{61}N_7O_{12}Na$ requires 830.4275).

(S)-*tert*-Butyl 2-((S)-2-((3S,6S,9S,12S,E)-6-isopropyl-3-(2-methoxy-2-oxoethylcarbamoyl)-9,9-dimethyl-5,8,11-trioxo-1,14-dioxa-4,7,10-triazacyclooctadec-16-en-12-ylcarbamoyl) pyrrolidine-1-carbonyl)pyrrolidine-1-carboxylate (30). Trifluoroacetate **28** (0.238 g, 0.388 mmol), *N*-*tert*-butoxycarbonyl L-prolyl L-proline **2** (0.122 g, 0.391 mmol) and HOBt hydrate (0.059 g, 0.39 mmol) were dissolved in CH_2Cl_2 (5 mL). *N*,*N*-Diisopropylethylamine (0.050 g, 0.39 mmol) dissolved in CH_2Cl_2 (1 mL) was added and the mixture cooled to 0 °C (ice bath). EDC hydrochloride (0.082 g, 0.43 mmol) was added in portions together with more CH_2Cl_2 (4 mL). The reaction mixture was stirred for 1 hour at 0 °C, after which the ice bath was removed and stirring continued for 24 h at room temperature. The volume was increased to 50 mL by addition of CH_2Cl_2 and the solution washed with 1 M aqueous H_2SO_4 (3 × 20 mL), 7.5% (w/w) K_2CO_3 solution (3 × 20 mL) and saturated brine (20 mL). The solution was dried with anhydrous $MgSO_4$ and the solvent evaporated affording the title compound as a white solid (0.251 g, 82%); δ_H (300 MHz; CD_2Cl_2) 7.95 (1H, s, N*H*(Aib)), 7.87 (1H, d, *J* 7, N*H*(Ser(All)$_1$)), 7.59 (1H, d, *J* 9, N*H*(Ser(All)$_2$)), 7.46 (1H, t, *J* 6, N*H*(Gly)), 6.94–6.80 (1H, d, *J* 6, N*H*(Val)), 5.89–5.68 (2H, m, C*H*=C*H*), 4.67–4.57 (2H, m, C$^\alpha$*H*(Ser(All)$_2$)/C$^\alpha$*H*(Ser(All)$_1$)), 4.41 (1H, m, C$^\alpha$*H*(Pro$_2$)), 4.35–4.29 (1H, m, C$^\alpha$*H*(Pro$_1$)),

4.12–3.82 (10H, m, $C^{\alpha}H$(Val)/$C^{\alpha}H_2$(Gly)/CH_2(Ser(All)$_1$)/CHH (Ser(All)$_2$)/CH_2CH=CH), 3.70–3.58 (3H, m, CHH(Ser(All)$_2$)/$C^{\delta}H_2$(Pro)), 3.67 (3H, s, OCH_3), 3.50–3.29 (2H, m, $C^{\delta}H_2$(Pro)), 2.45–2.26 (3H, m, CH_2(Pro)), 2.11–1.76 (6H, m, CH(CH_3)$_2$/CH_2(Pro)), 1.48–1.35 (15H, m, CH_3(Aib)/(CH_3)$_3$), 1.05–0.97 (6H, m, CH_3(Val)); δ_C (75 MHz; CD_2Cl_2) 177.0, 173.7, 172.4, 172.0, 171.3, 170.9, 170.4, 155.6, 133.1, 130.4, 81.6, 71.1, 70.0, 69.5, 66.9, 63.5, 63.2, 61.6, 58.0, 54.9, 54.5, 52.4, 47.8, 47.4, 41.7, 29.8, 29.4, 29.3, 28.6, 27.3, 26.7, 25.3, 23.6, 19.6, 17.7; *m/z* (ESI) 816.4103 ([M + Na]$^+$; $C_{37}H_{59}N_7O_{12}Na$ requires 816.4119).

***N-tert*-Butoxycarbonyl L-prolyl L-prolyl *O*-allyl L-seryl L-valyl L-valyl *O*-allyl L-seryl glycine methyl ester (31).** *O*-Allyl L-seryl L-valyl L-valyl *O*-allyl L-seryl glycine methyl ester trifluoroacetate **32** (0.524 g, 0.799 mmol), *N-tert*-butoxycarbonyl L-prolyl L-proline **2** (0.250 g, 0.800 mmol) and HOBt hydrate (0.122 g, 0.797 mmol) were dissolved in CH_2Cl_2 (5 mL). *N*,*N*-Diisopropylethylamine (0.103 g, 0.797 mmol) dissolved in CH_2Cl_2 (5 mL) was added. At this stage the reaction mixture turned into a gel. EDC hydrochloride (0.169 g, 0.882 mmol) was added in portions together with an additional 5 mL of CH_2Cl_2 and the reaction mixture vigorously stirred for 21 hours at room temperature. The volume was subsequently increased to 50 mL by addition of CH_2Cl_2. The solution was washed with 2 M aqueous H_2SO_4 (3 × 20 mL), 7.5% (w/w) K_2CO_3 solution (3 × 20 mL) and saturated brine (30 mL). The solution was dried with anhydrous $MgSO_4$ and the solvent evaporated affording the title compound as an off-white solid (0.489 g, 73%); δ_H (300 MHz; $CDCl_3$) 7.50–7.37 (3H, m, NH(Val$_x$)/NH(Gly)/NH(Ser(All)$_x$)), 7.12 and 6.85 (1H, d, *J* 6, NH(Ser(All)$_y$)), 7.02 (1H, d, *J* 5, NH(Val$_y$)), 5.89–5.71 (2H, m, CH=CH_2), 5.24–5.07 (4H, m, CH=CH_2), 4.83–4.76 (1H, m, $C^{\alpha}H$(Ser(All)$_x$)), 4.48–4.38 (3H, m, $C^{\alpha}H$(Ser(All)$_y$)/$C^{\alpha}H$(Pro$_x$)/$C^{\alpha}H$(Pro$_y$)), 4.31–4.27 (1H, m, $C^{\alpha}H$(Val$_y$)), 4.23–4.19 (1H, m, $C^{\alpha}H$(Val$_x$)), 4.09 (1H, dd, *J* 6 and 18, $C^{\alpha}H$H(Gly)), 3.99–3.92 (5H, m, C^{α}HH(Gly)/CH_2CH=CH_2), 3.87–3.37 (8H, m, CH_2(Ser(All)$_x$)/CH_2(Ser(All)$_y$)/$C^{\delta}H_2$(Pro$_x$)/$C^{\delta}H_2$(Pro$_y$)), 3.68 (3H, s, OCH_3), 2.34–1.81 (10H, m, CH_2(Pro$_x$)/CH_2(Pro$_y$)/CH(CH_3)$_2$), 1.44 and 1.36 (9H, s, (CH_3)$_3$), 1.02–0.85 (12H, m, CH_3); δ_C (75 MHz; $CDCl_3$) 172.8, 172.6, 171.9, 171.9, 171.8, 171.8, 171.7, 171.3, 171.1, 170.5, 170.4, 170.1, 170.1, 154.5, 153.3, 134.7, 134.6, 133.8, 133.6, 118.0, 117.6, 116.7, 116.6, 80.2, 79.7, 72.3, 72.0, 71.7, 69.5, 68.4, 62.1, 61.6, 61.0, 60.9, 60.1, 59.2, 57.7, 57.7, 55.1, 54.6, 53.2, 53.0, 52.0, 51.9, 47.3, 46.7, 46.5, 41.3, 30.6, 29.9, 29.8, 29.4, 28.9, 28.9, 28.4, 28.3, 25.5, 25.3, 24.5, 23.8, 19.4, 19.0, 18.9, 18.2, 18.1, 18.0, 17.9; *m/z* (ESI) 858.4575 ([M + Na]$^+$; $C_{40}H_{65}N_7O_{12}Na$ requires 858.4588).

***O*-Allyl L-seryl L-valyl L-valyl *O*-allyl L-seryl glycine methyl ester trifluoroacetate (32).** *N-tert*-Butoxycarbonyl *O*-allyl L-seryl L-valyl L-valyl *O*-allyl L-seryl glycine methyl ester **22** (0.734 g, 1.14 mmol) was dissolved in a 50% (v/v) solution of TFA in CH_2Cl_2 (16 mL). The reaction mixture was stirred for 1 h 30 min before the solvent and bulk of excess TFA were evaporated affording an oil. Addition of Et_2O (25 mL) resulted in the precipitation of a white solid. The mixture was centrifuged and the Et_2O decanted off. The residue was washed with an additional 2 × 25 mL Et_2O and dried under reduced pressure overnight affording a fine white powder (0.719 g, 96%); δ_H (300 MHz; d_6-DMSO) 8.48 (1H, d, *J* 9, NH(Val$_x$)), 8.41 (1H, t, *J* 6, NH(Gly)), 8.24 (3H, br s, NH_3^+), 8.01–7.98 (2H, m, NH(Val$_y$)/NH(Ser(All)$_2$)), 5.90–5.77 (2H, m, CH=CH_2), 5.30–5.10 (4H, m, CH=CH_2), 4.56–4.50 (1H, m, $C^{\alpha}H$(Ser(All)$_2$)), 4.32 (1H, dd, *J* 7 and 9, $C^{\alpha}H$(Val$_x$)), 4.24 (1H, dd, *J* 7 and 8, $C^{\alpha}H$(Val$_y$)), 4.10 (1H, br s, $C^{\alpha}H$(Ser(All)$_1$)), 3.98–3.93 (4H, m, CH_2CH=CH_2), 3.86–3.84 (2H, m, $C^{\alpha}H_2$(Gly)), 3.72–3.52 (4H, m, CH_2(Ser(All)$_1$)/CH_2(Ser(All)$_2$)), 3.61 (3H, s, OCH_3), 2.08–1.89 (2H, m, CH(CH_3)$_2$), 0.88–0.80 (12H, m, CH_3); δ_C (75 MHz; d_6-DMSO) 170.6, 170.4, 169.9, 169.8, 166.2, 158.3 (q, J_{CF} 37) 134.8, 134.4, 117.1, 116.9 (q, J_{CF} 293), 116.5, 71.4, 71.0, 69.5, 68.3, 57.9, 57.6, 52.4, 52.2, 51.6, 40.6, 30.6, 30.4, 19.1, 18.1, 17.9; *m/z* (ESI) 542.3181 (M$^+$; $C_{25}H_{44}N_5O_8$ requires 542.3189).

***N-tert*-Butoxycarbonyl L-prolyl L-prolyl *O*-benzyl L-seryl L-valyl L-valyl glycyl glycine methyl ester (33).** *O*-Benzyl L-seryl L-valyl L-valyl glycyl glycine methyl ester trifluoroacetate **34** (0.364 g, 0.573 mmol), *N-tert*-butoxycarbonyl L-prolyl L-proline **2** (0.179 g, 0.573 mmol) and HOBt hydrate (0.088 g, 0.58 mmol) were dissolved in CH_2Cl_2 (4 mL). *N*,*N*-Diisopropylethylamine (0.074 g, 0.57 mmol) dissolved in CH_2Cl_2 (1 mL) was added and the mixture cooled to 0 °C (ice bath). EDC hydrochloride (0.121 g, 0.631 mmol) was added in portions together with more CH_2Cl_2 (1 mL). The ice bath was removed after 1 hour and the reaction mixture stirred for 22 hours at room temperature. The volume was increased to 40 mL by addition of CH_2Cl_2 and the solution washed with 2 M aqueous H_2SO_4 (3 × 20 mL), 7.5% (w/w) K_2CO_3 solution (3 × 20 mL) and saturated brine (20 mL). The solution was dried with anhydrous $MgSO_4$ and the solvent evaporated affording the title compound as a slightly yellowish transparent/glassy solid (0.364 g, 78%); δ_H (300 MHz; $CDCl_3$) 7.56–7.51 (1H, m, NH(Val$_x$)), 7.48–7.37 (2H, m, NH(Gly$_x$)/NH(Gly$_y$)), 7.32–7.18 (6H, m, *Ph*/NH(Ser(Bn)), rotamer 1), 6.90 (1H, d, *J* 8, NH(Val$_y$)), 6.82 (1H, d, NH(Ser(Bn)), rotamer 2), 4.52-4.27 (6H, m, $C^{\alpha}H$(Val$_y$)/$C^{\alpha}H$(Ser(Bn))/$C^{\alpha}H$(Pro$_x$)/$C^{\alpha}H$(Pro$_y$)/CH_2Ph), 4.21-4.11 (1H, m, $C^{\alpha}H$(Val$_x$)), 4.07-3.85 (4H, m, $C^{\alpha}H_2$(Gly)), 4.06–3.75 (3H, m, CH_2(Ser(Bn))/$C^{\delta}H$H(Pro$_x$)), 3.73–3.58 (1H, m, $C^{\delta}H$H(Pro$_y$)), 3.64 and 3.62 (3H, s, OCH_3), 3.52–3.23 (2H, m, C^{δ}HH(Pro$_x$)/C^{δ}HH(Pro$_y$)), 2.49–2.36 (1H, m, CH(CH_3)$_2$), 2.34–1.91 (6H, m, CH(CH_3)$_2$/CH_2(Pro)), 1.87–1.49 (3H, m, CH_2(Pro)), 1.40 and 1.33 (9H, s, (CH_3)$_3$), 1.03–0.98 (6H, m, CH_3(Val$_x$)), 0.93–0.89 (6H, m, CH_3(Val$_y$)); δ_C (75 MHz; $CDCl_3$) 174.2, 173.2, 172.8, 172.5, 172.0, 172.0, 171.9, 171.8, 171.8, 171.8, 154.4, 153.1, 146.0, 137.2, 136.8, 128.4, 128.3, 128.1, 127.9, 127.8, 127.6, 80.3, 73.6, 73.1, 68.4, 62.2, 61.8, 61.6, 59.7, 58.7, 57.6, 55.7, 55.2, 53.4, 51.9, 51.8, 47.4, 47.3, 46.7, 46.4, 43.1, 40.9, 30.4, 29.4, 29.2, 29.1, 28.9, 28.3, 28.2, 25.6, 25.3, 24.4, 23.6, 19.5, 19.1, 19.0, 18.6, 18.3, 17.5; *m/z* (ESI) 838.4308 ([M + Na]$^+$; $C_{40}H_{61}N_7O_{11}Na$ requires 838.4326).

***O*-Benzyl L-seryl L-valyl L-valyl glycyl glycine methyl ester trifluoroacetate (34).** *N-tert*-Butoxycarbonyl *O*-benzyl L-seryl L-valyl L-valyl glycyl glycine methyl ester **24** (0.525 g, 0.844 mmol) was dissolved in a 50% (v/v) solution of TFA in CH_2Cl_2 (12 mL) at room temperature. The reaction mixture was stirred for 1 h 30 min before the solvent and bulk of excess TFA were evaporated. Et_2O (10 mL) was added to the liquid residue, which resulted in precipitation of a white solid. The Et_2O was decanted off and the residue washed with additional portions of Et_2O (2 × 10 mL) and dried under reduced pressure overnight affording the title compound as a white solid (0.494 g, 92%); δ_H (300 MHz; d_6-DMSO) 8.47 (1H, d, *J* 9, NH(Val$_x$)), 8.29–8.20 (5H, m, NH_3^+/NH(Gly$_x$)/NH(Gly$_y$)), 8.00 (1H, d, *J* 8, NH(Val$_y$))

7.38–7.27 (5H, m, *Ph*), 4.51 (2H, s, CH_2Ph), 4.34 (1H, dd, *J* 7 and 9, $C^{\alpha}H(Val_x)$), 4.17–4.12 (2H, m, $C^{\alpha}H(Val_y)/C^{\alpha}H(Ser(Bn))$), 3.88 (1H, dd, *J* 6 and 17, $C^{\alpha}HH(Gly_x)$), 3.81 (1H, dd, *J* 6 and 17, $C^{\alpha}HH(Gly_x)$), 3.76–3.64 (4H, m, $CH_2(Ser(Bn))/C^{\alpha}H_2(Gly_y)$), 3.62 (3H, s, OCH_3), 2.04–1.89 (2H, m, $CH(CH_3)_2$), 0.89–0.81 (12H, m, CH_3); δ_C (75 MHz; d_6-DMSO) 171.1, 170.6, 170.1, 169.2, 166.2, 158.3 (q, J_{CF} 32) 137.5, 128.2, 127.6, 127.5, 116.9 (q, J_{CF} 291), 72.5, 68.6, 58.1, 57.9, 52.2, 51.6, 41.6, 40.5, 30.6, 30.2, 19.1, 19.1, 18.2, 18.0; *m/z* (ESI) 522.2920 (M^+; $C_{25}H_{40}N_5O_7$ requires 522.2927).

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