Synthesis, Solution Structure and Biological Activity of Val-Val-Pro-Gln, a Bioactive Elastin Peptide[‡]

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Val-Val-Pro-Gln (valyl-valyl-prolyl-glutamine) is a small but highly conserved sequence present in all elastins. We describe its synthesis by mixed anhydride solution chemistry as an alternative to solid-phase peptide synthesis (SPPS). The molecular structure of the tetrapeptide in solution was investigated by classical spectroscopy, such as circular dichroism (CD), nuclear magnetic resonance (NMR) and Fourier Transform Infrared Spectroscopy (FTIR). The biological activity of Val-Val-Pro-Gln was evaluated by a bromodeoxyuridine (BrdU) incorporation assay with normal human dermal fibroblasts. This small peptide may play a critical role in control of matrix metabolism through its release from the elastin polypeptide chain during periods of tissue breakdown and remodelling.

Introduction

Elastin is a ubiquitous protein in all vertebrates. Elastin and type I collagen are among the most highly expressed proteins in mammalian systems.[1] Elastin is found in virtually all tissues, providing elasticity as well as contributing to hormonal control over matrix expression through its proteolysis and release of small peptides during remodelling.[2] One such small peptide is valyl-valyl-prolyl-glutamine (Val-Val-Pro-Gln), the subject of this report.

Elastin is produced from a single copy gene as a precursor protein, tropoelastin (750–800 residues),[3] which is deposited extracellularly and rendered insoluble through the formation of a unique tetrafunctional desmosine cross link.[4] The protein elastin is particularly abundant in blood vessels, lung tissue, skin and certain ligaments such as the paraspinous ligaments and the ligamentum nuchae of ruminants. It is a highly hydrophobic protein composed of over 90 % nonpolar amino acids and has one of the highest valine contents of any known protein (approaching 15 % in some species).[1]

Val-Val-Pro-Gln is a small but highly significant sequence of the elastin polypeptide chain. This sequence, located in the exon 8 coded domain, a lysine–proline-rich (KP) crosslinking domain, is highly conserved in all mammalian tropoelastins. It represents less than 1 % of the mass of the precursor chain, yet it appears to have hormonal effects, which control one or more aspects of matrix expression. It is presumably released from the elastin polypeptide chain through the action of matrix metalloproteinases (MMPs),[5,6] which are active in tissue remodelling. In purified elastin preparations it can be released through the action of the protease thermolysin, which cleaves on the N-terminal sides of amino acids with large hydrophobic side chains such as leucine, isoleucine and valine.[7]

Previous studies have identified important biological activities exhibited by small elastin peptides derived from thermolysin attack. A heptapeptide (LGAGGAG) and a related nonapeptide (LGAGGAGVL)[8] stimulate mRNA and cytokine production in fibroblast tissue cultures,[9] and other biological activities performed by these small peptides have also been identified. For instance, in vitro studies utilizing such thermolytic digests of elastin, containing Val-Val-Pro-Gln and other small peptides of elastin, have been found to increase the level of bromodeoxyuridine (BrdU) incorporation in normal human dermal fibroblasts (NHDF).[2] This increase in BrdU strongly suggests that stimulation of NHDF by Val-Val-Pro-Gln has mitogenic activity. It is probable that this response of cells to the elastin thermolysin-produced peptide mixture has important implications with respect to elastin synthesis in vivo.

[‡] Peptide VVPQ and certain compositions containing this peptide are covered by US Patent numbers 6069129, 6777389, 6794362 and worldwide counterparts.

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Accordingly, it is important to check the possibility that Val-Val-Pro-Gln is indeed able to increase mitogenic activity in normal human dermal fibroblasts. Therefore, we decided to synthesize Val-Val-Pro-Gln and to investigate its conformational properties.

Furthermore, the molecular structure of the tetrapeptide in solution has been investigated by classical spectroscopy, such as circular dichroism (CD), nuclear magnetic resonance (NMR), and Fourier transform infrared Spectroscopy (FTIR). Finally the mitogenic activity of Val-Val-Pro-Gln has been evaluated by bromodeoxyuridine (BrdU) incorporation assay in normal human dermal fibroblasts (NHDFs).

Results and Discussion

Peptide Synthesis

The chemical structure of Val-Val-Pro-Gln is shown in Figure 1.

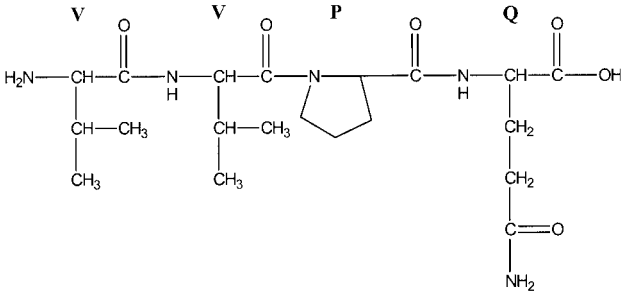


Figure 1. Chemical structure of Val-Val-Pro-Gln.

Solid-phase peptide synthesis (SPPS) of Val-Val-Pro-Gln by Fmoc chemistry was tried, but found to be difficult for several reasons. The penultimate proline is highly susceptible to diketopiperazine formation, resulting in reduced efficiency and very low yields.[10] In addition, when we tried to synthesize the tetrapeptide by SPPS, deprotection of the glutamine made recovery difficult without extensive HPLC purification schemes. Thus, synthesis of Val-Val-Pro-Gln by SPPS becomes an extremely costly and time-consuming endeavour, without any guarantee of significant purity. Furthermore, the necessity to produce gram amounts of this peptide for in-depth biological testing and industrial applications prompted us to develop a solution synthesis procedure. For these reasons, mixed anhydride solution synthesis, as described in this report, with use of Fmoc protection of amine groups, was developed as an alternative to SPPS for the synthesis of Val-Val-Pro-Gln.

Structural Studies

The CD spectra of Val-Val-Pro-Gln in water and in TFE at 0 °C and 25 °C are shown in Figure 2. In water the spectra at both temperatures are typical of dominantly unordered conformations. However, in the organic solvent there is the presence of a negative band at 222–224 nm and a positive band at 204–208 nm, which can be explained by several different interpretations. For example, different folded structures may be proposed, ranging from γ-turns[11,12] to β-turns.[13] In any case, because the spectra are also not significantly dependent on the temperature in this case, one may suggest that the turns are rather stable. By the way, it became necessary to implement conformational analysis by means of 2D ^{1}H NMR spectroscopy in TFE-d_3/H_2O (80:20).

The resonance assignment of the peptide was straightforwardly achieved by standard sequential assignment procedures[14] by use of combined analysis of 2D-TOCSY and 2D-NOESY spectra. TOCSY[15] spectra were used to identify spin systems, while NOESY[16] allowed the assignment of resonances to individual amino acids through sequential NOE connectivities. From the monodimensional ^{1}H NMR spectrum the presence of three different species was evident, pointing to *cis*- and *trans*-proline, because of an isomerization equilibrium, together with a third minor species assigned to the partial racemization of Val[2] during the 2+2 peptide coupling strategy. This species, according to NMR peak integration, represents 6 % of the total and may be neglected in the subsequent conformational analysis and

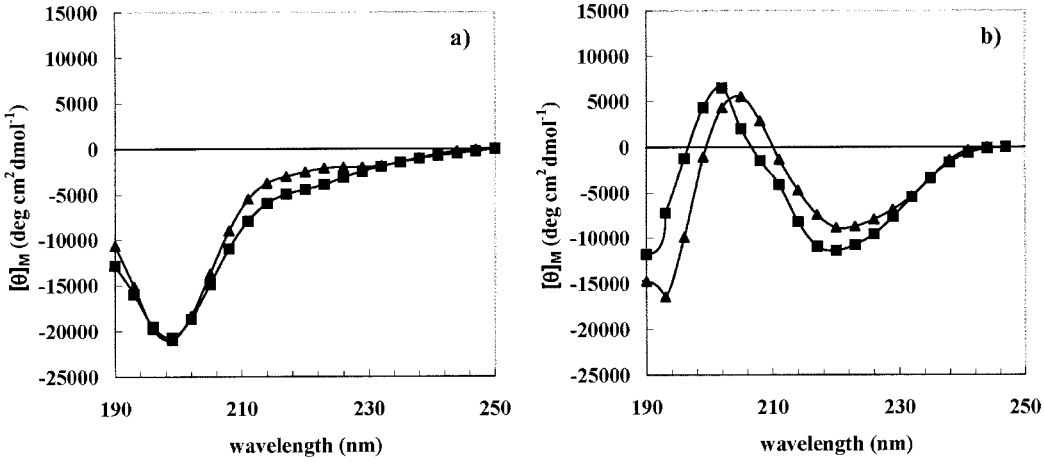


Figure 2. CD spectra of Val-Val-Pro-Gln recorded in water (a) and in TFE (b) at different temperatures: (■) 273 K, (▲) 298 K.

biological assays. The identification of the other two species was accomplished by NOE pattern analysis. The *trans*-proline conformation shows typical sequential $d_{\alpha\delta}$(Pro-1, Pro) NOEs, while the *cis* conformation presents $d_{\alpha\alpha}$(Pro-1, Pro) NOEs. Because the *trans* conformation is predominant (85 %), the following conformational analysis was carried out only for the *trans*-proline peptide. The chemical shifts are shown in Table 1, while a summary of other important NMR parameters is shown in Figure 3.

Table 1. Assignments of proton resonances of the peptide Val-Val-Pro-Gln in TFE-d_3/H_2O (80:20, v/v) at 298 K.

	Chemical shifts of proton resonances (ppm)			
Residue	NH	Hα	Hβ	Others
V^1	–	3.85	2.23	γ 1.03
V^2	7.92	4.59	2.16	γ 1.00/1.05
P^3	–	4.44	2.27/2.02	γ 2.12 δ 3.86/3.73
Q^4	7.81	4.45	2.25/2.03	γ 2.42 Nε 7.25/6.41

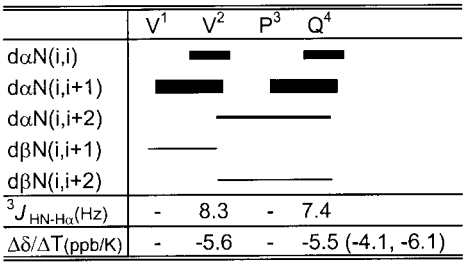


Figure 3. Summary of the NMR parameters of Val-Val-Pro-Gln peptide recorded in TFE-d_3/H_2O (80:20) at 283 K. The summary includes the temperature coefficients ($-\Delta\delta/\Delta T$, ppb K^{-1}, with the values for the Nε amide protons of the glutamine side chain indicated in brackets), the coupling constants ($^3J_{HN-H\alpha}$, Hz), and sequential and medium range NOEs. The NOE intensities are reflected by the thickness of the line

The NOE analysis suggested the presence of a folded conformation, as evidenced by nonsequential $d_{\alpha N}(i,i+2)$ and $d_{\beta N}(i,i+2)$ NOE connectivities between V2 and Q4. Because of the dimensions of the peptide, only β-turns and γ-turns can be regarded as folded conformations. Both types of secondary structures are usually, but not necessarily, stabilized by a hydrogen bond involving the backbone atoms. Nevertheless, the temperature coefficients for V2 and Q4 ruled out the presence of hydrogen bonds between the backbone amide groups. Interestingly, one proton of the side chain amide protons of glutamine showed a reduced temperature coefficient ($\Delta\delta/\Delta t$ = –4.1 ppb K^{-1}), suggesting the presence of a rather weak H-bond involving the Q4 side chain. However, while the donor of the hydrogen bond is easily determined by temperature coefficient analysis, the acceptor is harder to identify.

In order to produce a model of the conformation of Val-Val-Pro-Gln in solution, the NMR-derived constraints were used as input data for a simulated annealing structure calculation as implemented in a standard protocol in the CYANA program.[17] NOEs were translated into interproton distances and were used as the upper limit constraints, while $^3J_{HN-H\alpha}$ values were converted into angle constraints by application of the Karplus equation.[18] The CYANA calculation was performed with 50 randomized starting structures, and the 10 resulting conformers with the lowest target function were chosen for conformational analysis by the MOLMOL program.[19]

Figure 4 shows the backbone superposition of ten conformers with the lowest target function. The results indicated an S-shaped folded structure with an inverse γ-turn-like conformation in the V2–Q4 region. All the conformers show dihedral angle values in the range expected for an inverse gamma turn for the Pro residue ($\varphi = -75° \pm 10°$; $\psi = 65° \pm 10°$). This conformation is stabilized not by a main-chain-to-main-chain hydrogen bond, but more probably by a main-chain-to-side-chain hydrogen bond involving the side chain NHε proton of Q4 . The reduced number of NOEs involving the Q4 side chain protons precludes us from determining the preferred conformation for this side chain, so the identification of the H-bond acceptor was not possible.

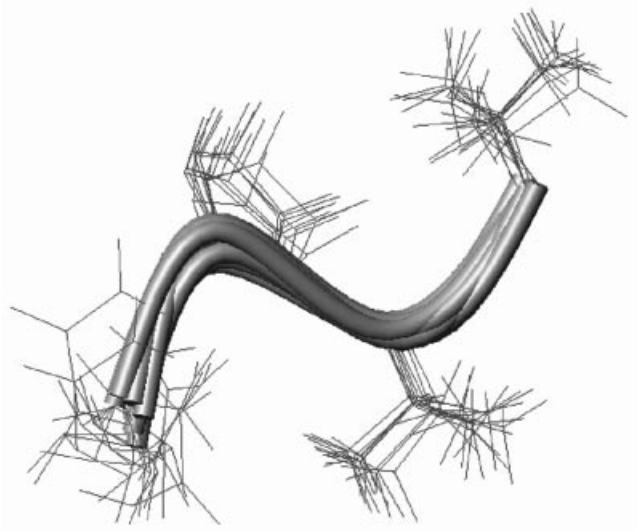


Figure 4. Structural model of Val-Val-Pro-Gln. Superposition of the backbone atoms (N, Cα, C′) of ten conformers with the lowest target function of peptide Val-Val-Pro-Gln. The backbone atoms are represented by grey ribbons, while the side chain atoms are represented by grey lines. (RMSD: 0.39 (±0.22) Å for backbone heavy atoms; 1.41 (±0.76) Å for all heavy atoms)

The FTIR absorption spectrum (Figure 5) appears to be in agreement with the NMR structural model, provided that the γ-turn is assumed to be solvated by trifluoroethanol. As a matter of fact, in the amide I region the band at 1633 cm^{-1} is assigned to the Val–Pro tertiary amide bond,[20] the lowering in frequency most probably being associated with hydrogen bonding to the solvent. Additional three bands are present at 1674, 1663 and 1616 cm^{-1}. The first is assigned to free C=O groups[21,22] and the second to the solvated amide carbonyls (mostly hydrogen-bonded to trifluoroethanol) γ-turn.[21] Finally, the third one could be assigned to the absorption of water, as suggested by an anonymous referee, although absorption of water is mostly found between 1640–1650 cm^{-1}.[23] However, because of the small intensity of the band, it could also originate from an

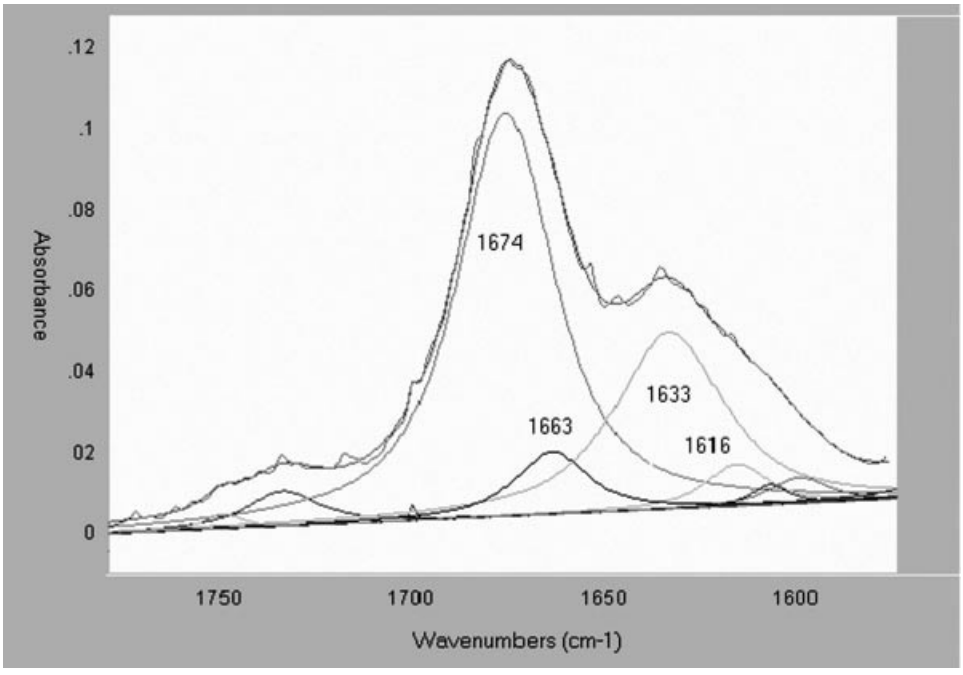


Figure 5. FT-IR absorption spectrum of Val-Val-Pro-Gln. Amide I region deconvoluted spectrum in TFE

artefact of decomposition, as suggested by another referee, and in any case is not significant for the assignment of the peptide conformation. Going on, these findings fit the NMR and CD data well in that they are not incompatible with the presence of an open γ-turn, devoid of any intramolecular H-bond, although solvated by trifluoroethanol.

Biological Activity Studies

Figure 6 shows the Val-Val-Pro-Gln and HEP-induced cellular activity as determined by the bromodeoxyuridine (BrdU) incorporation assay, tested in human dermal fibroblasts. It is easily seen that Val-Val-Pro-Gln increased the BrdU levels sixfold in relation to nonstimulated cells, whereas total HEP increased the BrdU levels threefold.

A more impressive image of BrdU incorporation in fibroblasts is given in Figure 7. The stimulation by total HEP and, even more so, by Val-Val-Pro-Gln is quite clear. HEP is a mixture of 42 discrete small elastin fragments, all of less than 1000 Da in mass.[2] These have been complete characterized as to composition (average size is five amino acids), but not for biologic activity, except for Val-Val-Pro-Gln as reported in this paper. By weight, Val-Val-Pro-Gln represents less than 1 % of the total peptides in HEP. Increased BrdU incorporation in stimulated cells, in relation to unstimulated controls indicates an increase in DNA synthesis. The increase in S-phase is coupled to an increase in cell division. This increased incorporation may indicate that Val-Val-Pro-Gln and HEP (partly through the action of its

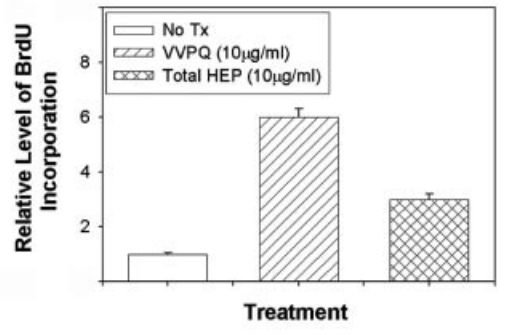


Figure 6. BrdU incorporation in normal human dermal fibroblasts. The bar graph represents the relative levels of BrdU incorporation after a 3-hour stimulation with the total mixture of hydrophilic elastin peptides (HEP) and Val-Val-Pro-Gln relative to the level of BrdU in nonstimulated controls. Val-Val-Pro-Gln increased BrdU levels by a factor of six in relation to nonstimulated levels, whereas total HEP increased the BrdU levels threefold. Six replicate values were averaged and the levels are shown plus/minus standard error. The differences were found to be significant by Student t-tests, $p < 0.001$.

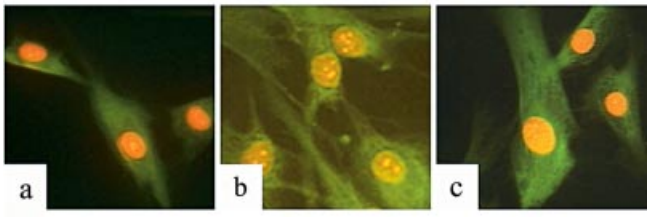


Figure 7. Image of BrdU incorporation in normal human dermal fibroblasts. The three-panel composite shows representative images of normal human dermal fibroblasts photographed after the 3-hour BrdU incubation period. The nuclei were stained red with propidium iodide (PI) and the BrdU labelled green with Alexa-488. The BrdU label and PI combined colour is yellow-orange in the nucleus, the cytoplasmic green is autofluorescence from the constitutive production of tropoelastin. Panel a) is nonstimulated cells, panel b) is Val-Val-Pro-Gln, and panel c) is total HEP.

Val-Val-Pro-Gln content) are mitogenic to normal dermal fibroblasts, causing them to increase their rate of proliferation. HEP has also been shown to increase the synthesis of tropoelastin in dermal fibroblasts,[2] but this capacity has not yet been tested with Val-Val-Pro-Gln. It may also be that there are other mitogen-inducing peptides in the HEP mixture that work with or without the action of Val-Val-Pro-Gln, but because the Val-Val-Pro-Gln effect was significantly more dramatic than that of HEP, this seems unlikely.

Conclusions

The results reported in this study demonstrate that this simple elastin-related tetrapeptide has significant biological/mitogenic activity toward human dermal fibroblasts in vitro. We believe this is diagnostic of a previously unappreciated role for elastin in matrix-related tissues: the hormonal control of matrix synthesis through release of peptides such as Val-Val-Pro-Gln during matrix remodelling and growth.

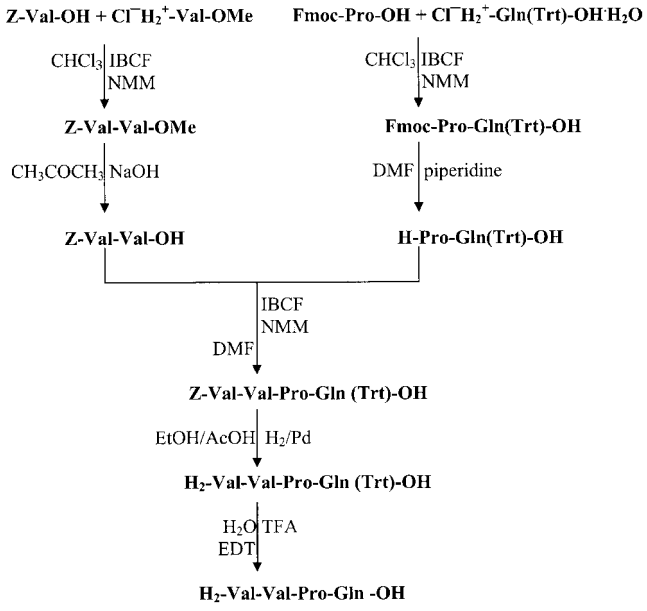
Our hypothesis is that the open γ-turn conformation ascertained for Val-Val-Pro-Gln in TFE is probably that adopted by the peptide when it is interacting with cellular receptors (the elastin binding protein?). Of course, this is not to assume a single molecular structure for a short tetrapeptide. Rather, although water strongly solvates the peptide backbone, thereby favouring extended, unordered conformations, TFE nevertheless mimics a less polar environment reasonably well, therefore selecting some relatively fixed structures, which are not discernible when exploring its conformational space in water by experimental techniques, because they are hidden within the conformational ensemble.

Finally, we would like to emphasize that this peptide may have useful applications in medicine (for instance, as a wound-healing preparation) and cosmetics. To this end, the solution synthesis reported here would be of value for large-scale production of the tetrapeptide Val-Val-Pro-Gln or of related peptides with the same C-terminal prolyl-glutamine sequence.

Experimental Section

List of Abbreviations: BrdU: bromodeoxyuridine; CCD: charge-coupled device. CD: circular dichroism spectroscopy; DMF: *N*,*N*-dimethylformamide; DMSO: dimethyl sulfoxide; DSS: 3-(trimethyl-silyl)propane-1-sulfonic acid; EDT: 1,2-ethanedithiol; Fmoc: 9-fluorenylmethoxy-carbonyl. FTIC: fluorescein-5-isothiocyanate; HEP: hydrophilic elastin peptides; HPLC: high-performance liquid chromatography; IBCF: isobutyl chloroformate; LSC: laser scanning cytometer; NHDF: normal human dermal fibroblasts; NMM: *N*-methylmorpholine; NMR: nuclear magnetic resonance; NOE: nuclear Overhauser enhancement; NOESY: NOE spectroscopy; P (or Pro): proline; PBS: phosphate-buffered saline; PBST: phosphate-buffered saline containing 0.05 % Tween-20; PI: propidium iodide; Q (or Gln): glutamine; TFA: trifluoroacetic acid; TFE: 2,2,2-trifluoroethanol; TOCSY: total correlation spectroscopy; TOF-MALDI/MS: time-of-flight, matrix-assisted laser desorption ionization mass spectrometry; Trt-: trityl; V (or Val): valine; Z: benzyloxycarbonyl; SPPS: solid-phase peptide synthesis.

General Remarks: Amino acids were purchased from Novabiochem AG (Laufelfinger, Switzerland). Purification of all synthetic products was performed by reversed-phase HPLC on a 250 mm × 4.6 mm micron Jupiter C-18 column with a binary gradient of water (0.1 % TFA) and acetonitrile (0.1 % TFA) as eluent. Subsequently the purity was ascertained by electrospray mass spectrometry. Further characterization was provided by ^{1}H NMR spectra recorded with a Bruker AMX 300 spectrometer. The synthesis of the peptide was performed as indicated in Scheme 1. Briefly, Fmoc and Z groups were used for N-terminal protection; these were removed throughout by use of piperidine and catalytic hydrogenolysis, respectively, while the coupling steps were accomplished by a classical mixed anhydride procedure. Finally, the deprotection steps were carried out.



Scheme 1.

Z-Val-Val-OMe: IBCF (13.0 mL, 99.5 mmol) was added at –13 °C to a solution of Z-Val-OH (25.0 g, 99.5 mmol) and NMM (10.9 mL, 99.5 mmol) in chloroform (70.0 mL). The temperature was maintained at –13 °C for 1 min, and Cl⁻H$_2$⁺-Val-OMe (16.7 g, 99.5 mmol) and NMM (10.9 mL, 99.5 mmol) were then added. The mixture was stirred at room temperature for 24 h. The organic solvent was removed under reduced pressure and the oily residue was dissolved in ethyl acetate and washed with 5 % sodium hydrogencarbonate, water, 5 % citric acid and water. The solution, dried with anhydrous sodium sulfate, was concentrated to dryness and the oil obtained was crystallized from ethyl acetate/petroleum ether to give a white solid (30.0 g, 84 % yield). ^{1}H NMR ([D$_6$]DMSO): δ = 8.11 (d, 1 H; NH Val^2), 7.40–7.23 (m, 6 H; H Ph and NH Val^1), 5.01 (s, 2 H; Ph-CH_2), 4.13 (m, 1 H; H$_\alpha$ Val), 3.96 (m, 1 H; H$_\alpha$ Val), 3.59 (s, 3 H; CH$_3$), 2.11–1.83 (m, 2 H; H$_\beta$ Val), 0.92–0.80 ppm (m, 12 H; H$_\gamma$ Val).

Z-Val-Val-OH: NaOH (1 N, 86.7 mL) was added with continuous stirring to a solution of Z-Val-Val-OMe (30.0 g, 82.4 mmol) in acetone (206.4 mL). After 3 h at room temperature, the organic solvent was evaporated under reduced pressure and water was added. The unchanged reagent was extracted with ethyl acetate, while the aque-

ous solution was cooled to 0 °C and neutralized by the dropwise addition of 1 N HCl (86.7 mL). The crude material was extracted with ethyl acetate and dried with anhydrous sodium sulfate. The solution was then concentrated to dryness and the remaining residue was crystallized from ethyl acetate/diethyl ether to give 26.7 g of crystalline solid (93 % yield). ^{1}H NMR ([D$_6$]DMSO): δ = 12.54 (br. s, 1 H; OH), 8.01 (d, 1 H; NH Val[2]), 7.39–7.22 (m, 6 H; H Ph and NH Val[1]), 5.00 (s, 2 H; Ph-CH_2), 3.97 (m, 2 H; H$_\alpha$ Val), 2.10–1.82 (m, 2 H; H$_\beta$ Val), 0.97–0.86 (m, 12 H; H$_\gamma$ Val).

Fmoc-Pro-Gln(Trt)-OH: IBCF (3.4 mL, 25.7 mmol) was added at –5 °C to a solution of Fmoc-Pro-OH (8.7 g, 25.7 mmol) and NMM (2.8 mL, 25.7 mmol) in chloroform (100.0 mL). The temperature was maintained at –5 °C for 1 min, and $Cl^-H_2^+$-Gln(Trt)-OH·H_2O (10.0 g, 25.7 mmol) and NMM (2.8 mL, 25.7 mmol) were then added. The mixture was continuously stirred at room temperature for 26 h. The organic solvent was removed under reduced pressure and the oily residue was dissolved in ethyl acetate and washed with 5 % sodium hydrogencarbonate, water, 5 % citric acid and water. The solution was dried with anhydrous sodium sulfate and concentrated to dryness, and the oil obtained was crystallized from ethyl acetate/diethyl ether to give the crystalline dipeptide (13.3 g, 73 % yield). ^{1}H NMR ([D$_6$]DMSO): δ = 12.58 (br. s, 1 H; OH), 8.50 (d, 1 H; NH Gln), 7.91–7.0 (m, 24 H; H Ph and NH$_\delta$ Gln), 4.42–3.88 (m, 6 H; H$_\alpha$ Pro, H$_\alpha$ Gln, H$_\delta$ P and CH$_2$), 2.29 (br. t, 2 H; H$_\gamma$ Gln), 1.98–1.62 (m, 6 H; H$_\beta$ Pro, H$_\gamma$ Pro and H$_\beta$ Gln), 1.07 ppm (t, 1 H; CH).

H-Pro-Gln(Trt)-OH: Piperidine (102.6 mL, 1.0 mol) was added to a stirred solution of Fmoc-Pro-Gln(Trt)-OH (12.8 g, 18.1 mmol) in *N*,*N*-dimethylformamide (200.0 mL). The reaction was monitored spectrophotometrically at λ = 301 nm for the absorbance of the free Fmoc group. After 2 h and 30 min at room temperature, the organic solvent was evaporated under reduced pressure and the oily residue was crystallized from methanol/diethyl ether to give 3.9 g of crystalline solid (45 % yield). ^{1}H NMR ([D$_6$]DMSO): δ = 12.57 (br. s, 1 H; OH), 8.49(d, 1 H; NH Gln), 7.88–6.7 (m, 16 H; H Ph and NH$_\delta$ Gln), 4.4–3.86 (m, 4 H; H$_\alpha$ Pro, H$_\alpha$ Gln, H$_\delta$ Pro), 2.3 (br. t, 2 H; H$_\gamma$ Q), 2.0–1.71 (m, 6 H; H$_\beta$ Pro, H$_\gamma$ Pro and H$_\beta$ Gln).

Z-Val-Val-Pro-Gln(Trt)-OH: IBCF (0.8 mL, 6.5 mmol) was added at –5 °C to a solution of Z-Val-Val-OH (2.3 g, 6.5 mmol) and NMM (0.7 mL, 6.5 mmol) in *N*,*N*-dimethylformamide (150.0 mL). The temperature was maintained at –5 °C for 1 min, and H-Pro-Gln(Trt)-OH (3.1 g, 6.5 mmol) and NMM (0.7 mL, 6.5 mmol) were then added. The mixture was stirred at room temperature for 24 h. The organic solvent was removed under reduced pressure and the oily residue was dissolved in ethyl acetate and washed with 5 % sodium hydrogencarbonate, water, 5 % citric acid and water. The solution was dried with anhydrous sodium sulfate and concentrated to dryness, and the oil obtained was crystallized from ethyl acetate/diethyl ether to give a white solid (3.8 g, 72 % yield). ^{1}H NMR ([D$_6$]DMSO): δ = 8.61 (d, 1 H; NH Gln), 8.12 (d, 1 H; NH Val), 7.80 (d, 1 H; NH Val), 7.40–7.11 (m, 21 H; H Ph and NH$_\delta$ Gln), 5.01 (s, 2 H; CH$_2$), 4.35–4.25 (m, 2 H; H$_\alpha$ Pro and H$_\alpha$ Gln), 4.10 (m, 1 H; H$_\alpha$ Val), 3.90 (m, 1 H; H$_\alpha$ Val) 3.70 (m, 1 H; H$_\delta$ Pro), 3.55 (m, 1 H; H$_\delta$ Pro), 2.39–2.25 (m, 2 H; H$_\gamma$ Gln), 2.0–1.65 (m, 8 H; H$_\beta$ Pro, H$_\gamma$ Pro, H$_\beta$ Gln and H$_\beta$ Val), 0.82 (m, 12 H; H$_\gamma$ Val).

H$_2$-Val-Val-Pro-Gln(Trt)-OH: Palladium/charcoal (Pd/C, 5 %, 49.5 mg) was suspended in 7.6 mL of ethanol/80 % aqueous AcOH (7:3) and the system was vigorously stirred under hydrogen for 30 min. Then a suspension of Z-Val-Val-Pro-Gln(Trt)-OH (210.0 mg, 0.27 mmol) in a minimal amount of the same solvent mixture was added. The reaction mixture was monitored until the evolution of CO_2 had stopped (about 3 h), and the mixture was maintained under nitrogen for an additional 15 min. The catalyst was filtered and repeatedly washed with ethanol/80 % aqueous AcOH (7:3). The solution obtained after filtration was evaporated under vacuum, water was then added, and the solution was lyophilized to remove any trace of unreacted products. The debenzyloxycarbonylated peptide was obtained in 60 % yield (113 mg). ^{1}H NMR ([D$_6$]DMSO): δ = 8.62 (d, 1 H; NH Gln), 8.22 (d, 1 H; NH Val), 7.43–7.12 (m, 16 H; H Ph and NH$_\delta$ Gln), 4.37–4.26 (m, 2 H; H$_\alpha$ Pro and H$_\alpha$ Gln), 4.12 (m, 1 H; H$_\alpha$ Val), 3.90 (m, 1 H; H$_\alpha$ Val) 3.71 (m, 2 H; H$_\delta$ Pro), 2.40–2.27 (m, 2 H; H$_\gamma$ Gln), 2.10–1.66 (m, 8 H; H$_\beta$ Pro, H$_\gamma$ Pro, H$_\beta$ Gln and H$_\beta$ Val), 0.83 ppm (m, 12 H; H$_\gamma$ Val).

H$_2$-Val-Val-Pro-Gln-OH: H$_2$-Val-Val-Pro-Gln(Trt)-OH (96 mg, 0.14 mmol) was dissolved in water (0.58 mL). The resulting solution was cooled in an ice bath, and EDT (0.24 mL) and TFA (9.7 mL) were added slowly.[24] The reaction mixture was stirred at 0 °C for few minutes and then at room temperature for 4 h. Following this incubation period, approximately 1 mL of dichloromethane was added and the solvent was partially evaporated under reduced pressure. Cold diethyl ether was then added to give a white solid (50 mg, 81 % yield). ^{1}H NMR ([D$_6$]DMSO): δ = 8.49 (d, 1 H; NH Gln), 8.39 (d, 1 H; NH Val), 7.53 (s, 1 H; NH$_\delta$ Gln), 6.82 (s, 1 H; NH$_\delta$ Gln), 4.38–4.27 (m, 2 H; H$_\alpha$ Pro and H$_\alpha$ Gln), 4.15 (m, 1 H; H$_\alpha$ Val), 3.99 (m, 1 H; H$_\alpha$ Val) 3.69 (m, 2 H; H$_\delta$ Pro), 2.40–2.30 (m, 2 H; H$_\gamma$ Gln), 2.22–1.97 (m, 8 H; H$_\beta$ Pro, H$_\gamma$ Pro, H$_\beta$ Gln and H$_\beta$ Val), 0.91 ppm (m, 12 H; H$_\gamma$ Val). Purification by HPLC and electrospray-mass spectrometry of the peptide are reported in Figure 8.

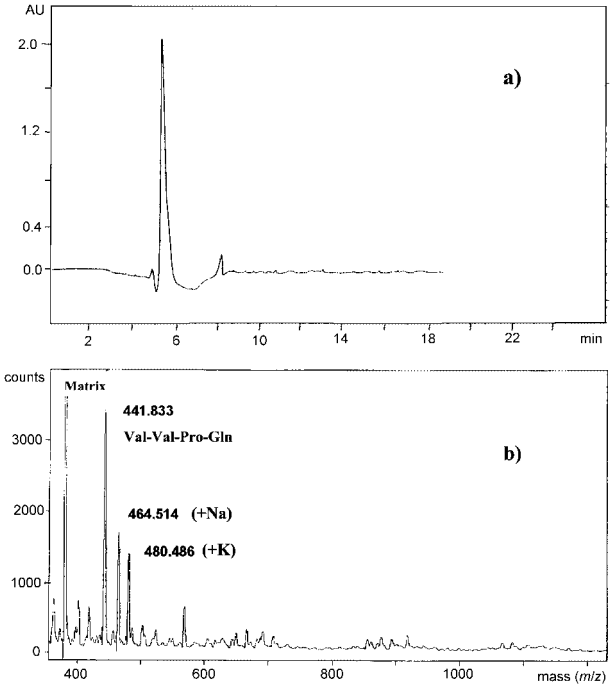


Figure 8. a) HPLC chromatogram of purified Val-Val-Pro-Gln. b) Electrospray-mass spectrometry of Val-Val-Pro-Gln.

Circular Dichroism Spectroscopy: CD spectra of 0.1 mg ml^{-1} solutions of peptides in water or trifluoroethanol were recorded on a JASCO J600 CD spectropolarimeter in a cell with a 1 mm optical path length with use of a HAAKE waterbath to control the temperature. Usually, 16 scans were acquired in the 190–250 nm range at temperatures of 273 K and 289 K, by taking points every 0.2 nm, with a 20 nm min^{-1} scan rate, an integration time of 2 s, and a 1 nm

bandwidth. Buffer solution was recorded under the same conditions and subtracted from the spectra before noise reduction. Smooth noise reduction was applied. The data are expressed as molar ellipticity $[\theta]_M$ in deg cm^2 dmol^{-1}.

Nuclear Magnetic Resonance Spectroscopy: All ^{1}H NMR experiments were performed on a Varian Unity INOVA 500 MHz spectrometer fitted with a 5 mm triple-resonance probe and *z*-axial gradients. The purified peptide was dissolved in 700 μL of TFE-d_3/H_2O (80:20), containing 0.1 mM of 3-(trimethylsilyl)propane-1-sulfonic acid (DSS) as internal reference standard at 0 ppm; 1.5 mM peptide solutions were used. One-dimensional ^{1}H spectra were acquired with 32 K datapoints with a sweepwidth of 6000 Hz. The residual HDO signal was suppressed by presaturation during the relaxation period. TOCSY[14] spectra were collected using 256 *t*1 increments and a spectral width of 6000 Hz in both dimensions. Relaxation delays were set to 2.5 s and spinlock (MLEV-17) mixing time was 80 ms. Shifted sine bell squared weighting and zero filling to 2 K × 2 K were applied before Fourier transformation. NOESY data were collected in essentially the same manner as for TOCSY data, with mixing times ranging from 200 to 400 ms. The residual HDO signal was suppressed by presaturation during relaxation time for TOCSY experiments, while for NOESY experiments Watergate pulse sequence were performed prior to acquisition.[25] Amide proton temperature coefficients were measured from 1D ^{1}H NMR spectra recorded in 5 K increments from 278 K to 318 K. Spectra were processed and analysed by VNMR Ver. 6.1C software (Varian, Palo Alto, CA).

Structure Calculation: Experimentally measured NOE intensities were converted into proton–proton distance constraints classified into three ranges: 1.8–2.7 Å, 1.8–3.3 Å and 1.8–5.0 Å, corresponding to strong, medium and weak NOEs, respectively. Pseudoatoms were introduced when no stereospecific assignment was determined, and interproton distances were corrected accordingly.[26] The two coupling constant of V2 and Q4, extracted from the 1D ^{1}H NMR spectrum, were used as angle constraints. The structures were calculated with the aid of the CYANA 1.0 program,[17] by use of a standard simulated annealing procedure. From an initial ensemble of 50 structures the best 10, in terms of target function values and residual distance restraint violations, were chosen to represent the conformations of the peptide. The resulting structures were analysed with the MOLMOL graphics program,[19] which was also used to produce the molecular model.

FTIR Spectroscopy: Infrared spectra at a resolution of 2 cm^{-1} were obtained in TFE solution with a Jasco FTIR spectrometer with a 0.025 cm cell with CaF_2 windows. The sample concentration was 50 mg mL^{-1}. The solvent spectrum obtained under identical conditions was subtracted from the sample spectrum. The amide I region of the spectra was decomposed into component bands by use of the software Grams/32® (Version 4.11 Level II Galactic Industries Corporation). The ratio between Lorentzian and Gaussian functions was fixed to the 2:8 ratio.

HEP-Induced Cellular Activity (Bromodeoxyuridine Incorporation Assay): Normal human dermal fibroblasts were purchased from Clonetics (BioWhittaker, Inc., Walkersville, MD) and maintained according to the specifications provided. The cells were growth expanded and samples were stored frozen in liquid nitrogen; cells were not utilized in experiments past passage 5. NHDFs were established in 6-well plates with 25 mm round glass coverslips and treated with 3 mM BrdU added simultaneously with 10 μg mL^{-1} of a total mixture of hydrolysed elastin peptides (HEP), 10 μg mL^{-1} Val-Val-Pro-Gln, PBS (negative control) or BrdU only (basal level of incorporation). The treated cells were incubated for 3 hours and were harvested by fixation in 70 % ethanol at –20 °C.[27] The fixed cells were incubated with *murine* monoclonal *anti*-BrdU (2 μg mL^{-1}) overnight at 4 °C. Excess *anti*-BrdU was removed by washing the coverslips in PBS containing 0.05 % Tween-20 (Sigma, St. Louis, MO) (PBST). The cells were then incubated with Alexa-488 conjugated goat *anti*-mouse secondary antibody (Molecular Probes, Eugene, OR) for 4 hrs at 22 °C, washed with PBST and counterstained for 10 min with propidium iodide (PI, 5 μg mL^{-1}) (Molecular Probes). The coverslips were protected with Permafluor (Fisher Scientific, Tustin, CA), placed onto microscope slides and dried flat in the dark, overnight.

FITC-labelled BrdU incorporation was quantified in replicate samples by use of a laser scanning cytometer (LSC) (CompuCyte, Cambridge MA)[27] The LSC is a microscope-based flow cytometer. Green fluorescent intensity reflected a quantitative measure of cellular activation as increased BrdU incorporation into S phase DNA in treated cells relative to control cells. To confirm the gating parameters, cells in selected gates were visually inspected through the CCD camera attached to the microscope. Optimized protocol and display settings were stored as configuration files and utilized for all samples within a given experimental set. An average of 5 000 ± 500 cells were scanned per treatment or condition.

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