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Synthesis and Biological Properties of the Seven Alanine-modified Analogues of the Marine Cyclopeptide Hymenamide C

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Abstract: The synthesis and biological activity of the marine cyclopeptide hymenamide C(1), showing an inhibitory effect on human neutrophil elastase degranulation release, were recently described. Based on this result, it was decided to undertake a systematic structure–activity relationship study of this cyclopeptide, based on the Ala-scan technique, in order to obtain useful information for the rational design of additional analogues. The synthesis and characterization of the seven Ala modified analogues are reported and their biological and pharmacological properties are described. Copyright © 2002 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Ala-scan technique; elastase degranulation; marine cyclopeptides; solid-phase synthesis

INTRODUCTION

We have recently reported the synthesis of the bioactive cyclopeptide hymenamide C [1] (**1**) (Figure 1) in order to investigate in more detail its ability to interfere with the immune system [2,3]. This compound was isolated from the marine sponge *Axinella carteri* collected off Vanuatu Island (South Pacific) [4].

The results of an extensive pharmacological screening showed that hymenamide C exerts an inhibitory effect on human neutrophil elastase degranulation release at micromolar levels [1]. This effect could potentially be used in a therapeutic approach to pulmonary emphysema [5] and to other adult respiratory distress syndromes [6]. With the aim of obtaining useful information for the design of novel therapeutics it was decided to undertake a systematic study of the structure–activity relationship by using the Ala-scan technique [7], a classical tool in peptide medicinal chemistry. This approach consists of the systematic replacement of each residue in the peptide sequence by Ala, in order to detect which side-chain functionality and conformational requirement are more favourable for the interaction with a specific receptor or a biomolecular target. As hymenamide C contains two Pro residues which, in

Abbreviations: AcOH, acetic acid; Boc, *tert*-butyloxycarbonyl; DCM, dichloromethane; DIEA, diisopropylethylamine; DKP, diketopiperazine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulphoxide; fMLP, HCO-L-Met-L-Leu-L-Phe-OH; Fmoc, 9-fluorenylmethoxycarbonyl; HATU, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium; HBTU, *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium; HOBt, 1-hydroxy-1,2,3-benzotriazole; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; NMM, *N*-methyl morpholine; PAC-PEG-PS, *p*-alkoxybenzyl alcohol-polyethylene glycol-polystyrene; PGE_2, prostaglandin E_2; PMA, phorbol-12-myristate-13-acetate; PyBop, benzotriazol-1-yl-tris-pyrrolidinophosphonium; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol.

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addition to the conformational restriction imposed by the cyclic structure, introduce a supplementary constraint to the peptide backbone, it would be very interesting to evaluate the relative importance of each Pro residue for the biological activity.

In this paper the synthesis, spectral properties and biological activities of the seven Ala-modified analogues of hymenamide C are reported.

MATERIALS AND METHODS

General Methods

Unless specified, solvents were reagent grade. They were purchased from Aldrich or Fluka (Milwaukee, WI) or LabScan (Stillorgan, Dublin, Ireland) and were used without further purification. DCM and DMF used for solid-phase reactions were synthesis grade (dried over 4 Å molecular sieves), and water and CH_3CN were HPLC grade. Fmoc-Glu(O^{γ}-PAC-PEG-PS)-O^{α}-allyl (Fmoc-Glu-O^{α}-allyl linked through the side chain to PAC-PEG-PS resin) was purchased from Perseptive Biosystems (Framingham, MA) (loading capacity 0.18 mmol/g), while the 2-chlorotritylchloride resin was from Novabiochem (Läufelfingen, Switzerland) (loading capacity 1.08 mmol/g). The Fmoc-L-amino acids and the coupling reagents (HOBt, HBTU, PyBop and HATU) were supplied by Novabiochem or Fluka and used without further purification. Solid-phase reactions were carried out on batch, in a glass reaction vessel or on a polypropylene ISOLUTE SPE column on a VAC MASTER system (a manual parallel synthesis device purchased from Stepbio, Bologna, Italy) and using the Fmoc/Boc/allyl protocol. For quantification of the Fmoc amino acids on the resin,

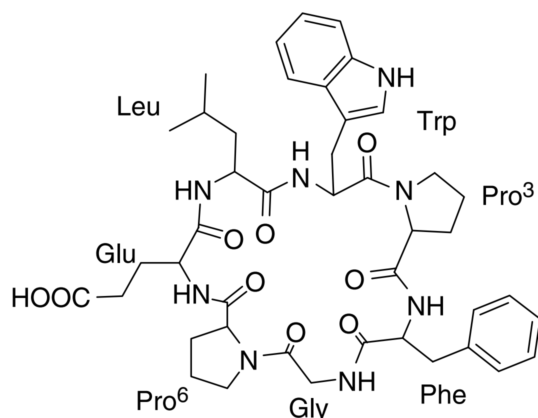

Figure 1 Hymenamide C (*cis*-Pro^3, *trans*-Pro^6) (**1**).

absorbance at 301 nm was read employing a Shimadzu UV 2101 PC spectrophotometer. The ^{1}H and ^{13}C (^{1}H–^{1}H and ^{1}H–^{13}C) spectra were recorded using a Bruker Avance 600 MHz spectrometer with a deuterated solvent (CD_3OD or d_6-DMSO). The ^{1}H NMR temperature coefficients experiments were conducted at 27°, 30°, 35°, 40°, 45°, 50°, 55° and 60 °C. The LCQ Thermoquest mass spectrometer was used to record the ESMS spectra.

General Procedures for Solid-Phase Reactions

a. 2-Chlorotritylchloride resin loading. Fmoc-Ala-OH (0.5 eq), DIEA (2 eq) in DCM (10 ml/g of resin), 2 h; capping with 20 ml of DCM, MeOH, DIEA (17 : 2 : 1); washings (1.5 min each): 3 × 3 ml of DCM, 2 × 3 ml of DMF, 2 × 3 ml of DCM.

b. Fmoc deprotection. 20% piperidine in DMF (3 ml), 1.5 min; 3 ml of 20% piperidine in DMF, 10 min; washings (1.5 min each): 2 × 3 ml of DMF, 3 × 3 ml of DCM, 2 × 3 ml of DMF.

b1. Fmoc deprotection. 20% piperidine in DMF (3 ml), 5 min; washings (1.5 min each): 2 × 3 ml of DMF, 3 × 3 ml of DCM, 2 × 3 ml of DMF.

c. Ala^1-Ala^6 (2–7) peptide coupling conditions. HOBt (4 or 5 eq), HBTU (4 or 5 eq), Fmoc-amino acid (4 or 5 eq), NMM (5 or 6 eq) in 2.5 ml of DMF, 2 h; washings (1.5 min each): 3 × 3 ml of DMF, 3 × 3 ml of DCM.

c1. Ala^7 (8) peptide coupling conditions. HOBt (4 eq), HBTU (4 eq), Fmoc-amino acid (4 eq), NMM (5 eq) in DMF (500 µl/100 mg of resin), 2 h; washings (1.5 min each): 3 × 3 ml of DMF, 3 × 3 ml of DCM.

d. Spectrophotometric analysis of the Fmoc chromophore. The assay was performed on duplicate samples. 0.4 ml of piperidine and 0.4 ml of DCM were added to two dried samples of the resin-bound peptide (~6 mg) in two 10 ml volumetric flasks. The reaction was allowed to proceed for 30 min at room temperature in the sealed flasks. 1.6 ml of MeOH was added and the solutions were diluted to 10 ml volume with DCM. A reference solution was prepared in a 10 ml volumetric flask using 0.4 ml of piperidine, 1.6 ml of MeOH and DCM to volume. The solutions were shaken and the absorbance of the samples *versus* the reference solution was measured at 301 nm. The substitution degree (in mmol of amino acid/g of resin) was calculated from the equation: mmol/g = $(A_{301}/7800) \times (10$ ml/g of resin$)$.

e. Ala^1-Ala^6 analogues (2–7) mini-cleavage conditions. After drying the resin-bound peptide

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under vacuum for 1 h, a small aliquot (~18 mg) was removed and treated with a solution of TFA/H_2O (95 : 5; 100 µl × 1 mg of resin) for 1 h under stirring. The mixture was filtered through a small plug of cotton-wool in a Pasteur pipette, rinsed with a small volume of TFA 95% and collected in a centrifuge tube. The filtrate was reduced to minimum volume (~500 µl) with a nitrogen stream and cold diethyl ether (20 ml × 1 ml of filtrate) was added. The white precipitate was kept at 0 °C for 2 h. The peptide was collected by centrifugation, washed with cold diethyl ether, suspended in water and, after lyophilization, analysed by RP-HPLC to determine the success of the reaction by the relative intensity of the peaks assigned to protected and deprotected peptide.

e1. Ala^7 analogue (8) mini-cleavage conditions. After drying the resin-bound peptide under vacuum for 1 h, a small aliquot (~18 mg) was removed and treated with a solution of AcOH/TFE/DCM (2 : 2 : 6; 100 µl × 1 mg of resin) for 2 h under stirring. The resin was removed by filtration through a small plug of cotton-wool in a Pasteur pipette, rinsed three times with a small volume of solution and collected in a falcon tube. Hexane (15 times volume) was added and evaporated to remove AcOH as an azeotrope with hexane. The linear Boc-protected heptapeptide was lyophilized, weighed and analysed by RP-HPLC to determine the success of the reaction.

Synthesis of the Terminally Deprotected Linear Anchored Ala^1–Ala^6 Heptapeptides (2–7)

Fmoc-Glu(O^γ-PAC-PEG-PS)-O^α-allyl (0.148 mmol/g medium loading level) was placed into a 50 ml reaction glass vessel and allowed to swell for 45 min with 3 ml of DMF. It was Fmoc-deprotected according to general procedure **b**. The resin was subsequently submitted to the following series of six coupling-deprotection cycles: (i) peptide coupling according to general procedure **c** with appropriate amino acid, followed by Fmoc deprotection according to general procedure **b1** to avoid the formation of DKP. The result of the Fmoc removal was monitored according to general procedure **d**; (ii) peptide coupling according to general procedure **c** with appropriate amino acid, followed by determination, according to general procedure **d**, of the degree of substitution of the resin bound peptide. As the DKP formation was low, the Fmoc deprotection was performed according to general procedure **b**; (iii–v) peptide coupling according to general procedure **c** with appropriate amino acid, followed by Fmoc deprotection according to general procedure **b**; (vi) peptide coupling according to general procedure **c** with appropriate amino acid.

The ninhydrin test was performed after each amino acid coupling step and the coupling repeated if necessary. The resin was then removed from the glass reaction vessel and dried under vacuum for 1.5 h. After weighing, the dried resin-bound peptide was submitted to treatment with $Pd^0(PPh_3)_4$ (4 or 5 eq) in $CHCl_3$–AcOH–NMM (37 : 2 : 1) (mechanical shaking overnight at room temperature and under argon) to remove the allyl protecting group at the *C*-terminus of the peptide anchored to the resin. The resin was washed with 70 ml of DIEA/DMF (0.5 : 99.5), 70 ml of sodium diethyldithiocarbamate in DMF (0.5 : 99.5 w/w) and the *N*-deprotected linear anchored heptapeptide was subsequently obtained by removing the Fmoc protecting group at the *N*-terminus of the sequence (20% piperidine in DMF).

On-Resin Cyclization of the Terminally Deprotected Linear Anchored Ala^1–Ala^6 Heptapeptides (2–7)

Cyclization was performed on solid-phase by 4 or 5 eq of HOBt, 4 or 5 eq of HBTU and 8 or 10 eq of NMM in 2.5 ml of DMF for 5.5 h, at room temperature, under mechanical shaking. The cyclization reaction was monitored by the ninhydrin test. Only the Ala^6 analogue required a second step, using 8 eq of HOBt, 8 eq of PyBop and 10 eq of DIEA for 5.5 h, at room temperature, under mechanical shaking.

Cleavage from Resin and N^{in}-Boc-Deprotection for the Ala^1-Ala^6 Heptapeptides (2–7)

The support-bound peptide was washed with DMF and DCM and then dried. The resin-bound peptide was Trp^{in}-deprotected from Boc and cleaved from the solid support by treatment with TFA/H_2O (95 : 5) for 1 h under stirring. The cleavage mixture was filtered off and the resin was washed three times with 95% TFA (6 ml). The filtrate volume was reduced under a nitrogen stream and then a 20-fold excess of cold diethyl ether was added. A white precipitate appeared soon after the diethyl ether addition. For maximum recovery, the diethyl ether–peptide suspension was kept at 0 °C for 2 h. The precipitated peptide was collected by filtration through a 0.45 µm PTFE membrane filter (PALL Corporation, Ann Arbor, MI) installed on a glass 47 mm

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filter holder with a vacuum aspirator. The precipitate was washed with cold diethyl ether. The filter was transferred to a glass vessel, the solid removed by water with few drops of glacial AcOH and ultrasound, and the suspension was lyophilized. The following amounts of crude cyclopeptides and overall yields (calculated on the basis of the resin loading, as determined by UV analysis according to procedure **d**) were obtained: Ala^1 analogue **(2)**: 52.3 mg, 78.4%; Ala^2 analogue **(3)**: 59.3 mg, 92.5%; Ala^3 analogue **(4)**: 50.3 mg, 62.8%; Ala^4 analogue **(5)**: 62.8 mg, 93%; Ala^5 analogue **(6)**: 39.2 mg, 51.8%; Ala^6 analogue **(7)**: 30.8 mg, 44.1%.

HPLC Analysis and Purification of the Ala^1–Ala^6 Heptapeptides (2–7)

The crude cyclopeptides were analysed by RP-HPLC on a Jupiter C-18 analytical column (250 × 4.60 mm, 5 μm, 300 Å), using a 40 min gradient from 25 : 75% to 50 : 50% of CH_3CN/H_2O (each containing 0.1% TFA) at a flow rate of 1.0 ml/min and UV detection at 210 nm. The HPLC analysis showed: (a) two main peaks of different intensity relative to *cis*/*trans* isomers of the synthetic Ala^1 and Ala^5 analogues, respectively; (b) a main peak relative to the synthetic Ala^2, Ala^3, Ala^4 and Ala^6 analogues, respectively. The crude cyclopeptides were then purified by semi-preparative RP-HPLC on a Jupiter C-18 column (250 × 10.00 mm, 10 μm, 300 Å), using the same elution conditions as those of the analytical HPLC at a flow rate of 5.0 ml/min, with UV detection at $\lambda = 230$ nm for the Ala^2 analogue, and at $\lambda = 300$ nm for the other Ala analogues. The HPLC purification yielded, as white solids, the *cis*/*trans* Ala analogues, identified on the basis of ESMS and ^{1}H NMR experiments.

Synthesis of the Ala^7 Analogue *c*-(Leu-Trp-Pro^3-Phe-Gly-Pro^6-Ala) (8)

2-Chlorotritylchloride resin (600 mg, 1.08 mmol/g loading level) was placed into a 25 ml polypropylene ISOLUTE COLUMN on a VAC MASTER system, swelled for 1 h with 3 ml of DMF by a nitrogen stream and then washed with 2 × 3 ml of DCM. The resin was loaded with Fmoc-Ala-OH (100.86 mg, 0.324 mmol), in order to obtain a lower substitution level, according to procedure **a**. Unreacted trityl groups were capped with methanol. The resin was dried under vacuum over KOH and the resulting substitution level (0.497 mmol/g) was determined spectrophotometrically according to the general procedure **d**. After Fmoc-Ala-*O*-2ClTrt-Cl swelling (45 min with 3 ml of DMF), the Fmoc protecting group was removed according to general procedure **b**. The resin was subsequently submitted to the following series of six coupling–deprotection cycles: (i) peptide coupling according to procedure **c1** with Fmoc-L-Pro-OH (402.2 mg, 1.19 mmol), HOBt (182.5 mg, 1.19 mmol), HBTU (452.12 mg, 1.19 mmol) and NMM (163.8 μl, 1.5 mmol), followed by Fmoc deprotection according to general procedure **b1** to avoid the formation of DKP. The extent of Fmoc removal was monitored according to general procedure **d**; (ii) peptide coupling according to general procedure **c1** with Fmoc-L-Gly-OH (354.4 mg, 1.19 mmol), HOBt (182.5 mg, 1.19 mmol), HBTU (452.12 mg, 1.19 mmol) and NMM (163.8 μl, 1.5 mmol), followed by determination, according to general procedure **d**, of the level of substitution of the resin-bound peptide. As the DKP formation was low, the Fmoc deprotection was performed according to general procedure **b**; (iii–v) peptide coupling according to general procedure **c1** with Fmoc-L-Phe-OH, Fmoc-L-Pro-OH and Fmoc-L-Trp(Boc)-OH successively (1.19 mmol), HOBt (182.5 mg, 1.19 mmol), HBTU (452.12 mg, 1.19 mmol) and NMM (163.8 μl, 1.5 mmol), followed by Fmoc deprotection according to general procedure **b**; (vi) peptide coupling according to general procedure **c1** with Fmoc-L-Leu-OH (421.3 mg, 1.19 mmol), HOBt (182.5 mg, 1.19 mmol), HBTU (452.12 mg, 1.19 mmol) and NMM (163.8 μl, 1.5 mmol).

The linear anchored N^{in}-Boc protected heptapeptide was submitted to Fmoc deprotection according to general procedure **b**. The resin was removed from the polypropylene column, washed with methanol and dried under vacuum over KOH for 1 h. After weighing the dried resin-bound peptide, a sample was collected and submitted to mini-cleavage according to general procedure **e1** to determine the success of the reaction. The overall resin-bound peptide was cleaved from the solid support by treatment with an AcOH/TFE/DCM (2 : 2 : 6) solution for 2 h under stirring. The cleavage mixture was filtered off and the resin was washed three times with the same solution. Hexane was added (15 times volume) and the solution was evaporated, adding further hexane if necessary. The crude peptide product appeared as a green-yellow sticky oil, which was lyophilized, yielding 323.3 mg of linear N^{in}–Boc protected heptapeptide. It was analysed by RP-HPLC on a Jupiter C-18 analytical column (250 × 4.60 mm, 5 μm, 300 Å), using a 40 min gradient from 25 : 75%

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to 50 : 50% of CH_3CN/H_2O (each containing 0.1% TFA) at a flow rate of 1.0 ml/min and UV detection at 210 nm. The HPLC analysis showed one peak that was identified as the N^{in}–Boc protected Ala[7] analogue on the basis of an ESMS experiment. The crude linear N^{in}–Boc protected heptapeptide was then purified by semi-preparative RP-HPLC on a Jupiter C-18 column (250 × 10.00 mm, 10 μm, 300 Å), using the same elution conditions as those of the analytical HPLC at a flow rate of 5.0 ml/min, with UV detection at $\lambda = 300$ nm. The HPLC purification yielded 224 mg of the linear N^{in}–Boc protected Ala[7] analogue (0.252 mmol, 84.7% yield based on a resin loading of 0.497 mmol/g, as determined by UV analysis according to procedure **d**, $R_t = 31.58$ min; ESMS, m/z 887.3 for $[M + H]^+$) that was dissolved in 366 ml of DCM with HATU (189.7 mg, 0.499 mmol) and DIEA (88.5 μl, 0.63 mmol). The solution was stirred for 1 h on an ice bath and then allowed to warm at room temperature and kept at this temperature for 24 h. During this time the cyclization reaction was monitored via HPLC. After 24 h the solvent was removed, yielding 297.4 mg of crude N^{in}-Boc protected cyclopeptide ($R_t = 43.50$ min; ESMS, m/z 869.30 for $[M + H]^+$). N^{in}-Boc deprotection was obtained by treatment with 95% TFA in water (100 μl × 1 mg of resin) for 1 h under stirring. The cleavage mixture was evaporated and lyophilized. The crude cyclopeptide was analysed by RP-HPLC on a Jupiter C-18 analytical column as previously described. The HPLC analysis showed two main peaks of different intensity identified as the *cis/trans* isomers of the synthetic Ala[7] analogue on the basis of ESMS and ^1H NMR experiments. The crude cyclopeptides were then purified by semi-preparative RP-HPLC using the same elution conditions as those used for theN^{in}-Boc protected heptapeptide. The HPLC purification yielded successively, as white solids, the *cis*^{3}-*trans*^{6} Ala[7] analogue and the *cis*^{3}-*cis*^{6} Ala[7] analogue.

Preparation of Human Neutrophils

The citrated blood of healthy volunteers was centrifuged at 200***g*** for 15 min at room temperature. The platelet-rich plasma was removed and the leukocytes contained in the residual blood were isolated by sedimentation with 2% w/v dextran in 0.9% NaCl at room temperature for 45–60 min. The upper phase was then collected and concentrated by centrifugation at 200***g*** for 10 min at room temperature. Contaminating erythrocytes were lysed by hypotonic treatment using ice-cold distilled water for 20 s. The cell pellets were gently resuspended in 10 ml of ice-cold modified phosphate buffer saline (free of Ca^{2+} and Mg^{2+} ions) and a solution of Ficoll-hypaque was carefully layered under the cell mixture to form a discontinuous gradient. The cell gradient mixture was centrifuged at 400***g*** for 40 min at room temperature. Neutrophils were separated and resuspended in phosphate buffer saline containing 1.26 mM Ca^{2+} and 0.9 mM Mg^{2+} ions [8]. Viability was greater than 95% by the trypan blue exclusion test.

Cell Viability Assays

The mitochondrial dependent reduction of MTT bromide to formazan [9] was used to assess the possible cytotoxic effect of the Ala-modified analogues on human neutrophils as well as on the mouse macrophage cell line RAW 264.7.

Elastase Release by Human Neutrophils

Aliquots of 1.0 ml human neutrophils (2.5×10^6 cells/ml) were preincubated at 37 °C for 5 min with 10 μl of the Ala-modified analogues dissolved in ethanol (or of an equivalent volume of ethanol for the controls). Then, the suspensions were stimulated for a further 10 min at 37 °C using as stimuli cytochalasin B (10 μM) and fMLP (10 nM). Elastase activity was estimated in aliquots of stimulated human neutrophil supernatants incubated with *N*-*tert*-butoxycarbonyl-L-alanine *p*-nitrophenyl ester. The extent of *p*-nitrophenol release was measured at 414 nm on a microplate reader. The direct effects on elastase were also tested using aliquots of supernatants of cytochalasin B + fMLP-stimulated human neutrophils [10].

Chemiluminescence

The chemiluminescence generated by human neutrophils stimulated with PMA (1 μM) in the presence of luminol (40 μM) was recorded in a Microbeta Trilux (Wallac, Turku, Finland) [11] counter.

Cell Culture

The mouse macrophage cell line RAW 264.7 (European Collection of Cell Cultures) was cultured in Dulbecco's modified Eagle's medium containing 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum. Macrophages were removed from the tissue culture flask using a cell scraper and centrifuged at 800***g*** for 10 min. The cells were resuspended

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at a concentration of 2×10^6/ml in a total volume of 200 μl and cultured in 96-well culture plates. Macrophages were co-incubated with the Ala-modified analogues and *Escherichia coli* LPS (serotype 0111: B4) (10 μg/ml) at 37 °C for 18 h. Prostaglandin E_2 and nitrite production were determined in 18 h supernatants.

Statistical Analysis

The results are presented as mean ± SEM; *n* represents the number of experiments. 50% inhibitory concentration (IC_{50}) values were calculated from at least four significant concentrations. The level of statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons.

RESULTS AND DISCUSSION

Cyclopeptides **2–7** (Table 1) were synthetized on solid phase using a three-dimensional, orthogonal protection scheme (Fmoc/Boc/allyl) and a 'by side-chain' anchorage on resin of the *C*-terminal amino acid residue as previously described for the parent compound [1]. Ala-derivatives **2–7** were first synthetized as linear precursors and subsequently cyclized on the resin. All the Fmoc-protected amino acids were activated by HOBt/HBTU in the presence of NMM and the progress of the couplings was checked by the Kaiser test. Fmoc deprotection was obtained by treatment with a piperidine solution. After the synthesis of the linear heptapeptides was accomplished, deprotection at *C*- and *N*-termini was performed. The cyclization step was allowed to proceed on the resin with HOBt/HBTU activation

Table 1 Sequences of Hymenamide C and Its Ala-Modified Cyclopeptides along with the Conformations at Xxx-Pro Peptide Linkages

			Major conformer	Minor conformer
1	Hymenamide C	*cyclo*-[Leu-Trp-Pro^3-Phe-Gly-Pro^6-Glu]	*cis*^{3}-*trans*^{6}	*cis*^{3}-*cis*^{6}
2	Ala^1 analogue	*cyclo*-[Ala-Trp-Pro^3-Phe-Gly-Pro^6-Glu]	*cis*^{3}-*trans*^{6}	*cis*^{3}-*cis*^{6}
3	Ala^2 analogue	*cyclo*-[Leu-Ala-Pro^3-Phe-Gly-Pro^6-Glu]	*cis*^{3}-*trans*^{6}	—
4	Ala^3 analogue	*cyclo*-[Leu-Trp-Ala-Phe-Gly-Pro^6-Glu]	*trans*^{6}	—
5	Ala^4 analogue	*cyclo*-[Leu-Trp-Pro^3-Ala-Gly-Pro^6-Glu]	*cis*^{3}-*trans*^{6}	—
6	Ala^5 analogue	*cyclo*-[Leu-Trp-Pro^3-Phe-Ala-Pro^6-Glu]	*cis*^{3}-*trans*^{6}	*cis*^{3}-*cis*^{6}
7	Ala^6 analogue	*cyclo*-[Leu-Trp-Pro^3-Phe-Gly-Ala-Glu]	*cis*^{3}	—
8	Ala^7 analogue	*cyclo*-[Leu-Trp-Pro^3-Phe-Gly-Pro^6-Ala]	*cis*^{3}-*trans*^{6}	*cis*^{3}-*cis*^{6}

Table 2 Most Significant Analytical Data for Hymenamide C and Its Ala-Modified Cyclopeptides

		HPLC t_R (min)		Isomer ratio (mg)		Mass data[a]	
		Major conformer	Minor conformer	Major conformer	Minor conformer	Major conformer	Minor conformer
1	Hymenamide C	32.31	30.96	3.6	2.2	827.0	827.0
2	Ala^1 analogue	16.70	15.37	7.2	2.7	785.4	785.3
3	Ala^2 analogue	13.10	—	10.2	—	712.5	—
4	Ala^3 analogue	22.42	—	5.5	—	801.3	—
5	Ala^4 analogue	12.24	—	9.2	—	751.5	—
6	Ala^5 analogue	26.85	25.01	4.5	1.7	841.6	841.6
7	Ala^6 analogue	23.70	—	5.8	—	801.3	—
8	Ala^7 analogue	26.10	23.40	76.3	7.0	769.4	769.4

[a] ESMS, m/z for $[M + H]^+$.

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Table 3 ^{1}H NMR[a] Data for the Ala-scan *Major* Analogues

	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	$C^{o}H$	$C^{m}H$	$C^{p}H$	$C^{2}H$	$C^{4}H$	$C^{5}H$	$C^{6}H$	$C^{7}H$
Ala^{1}												
Ala	4.59	1.38										
Trp	4.47	3.26/3.24						7.16	7.36	7.11	6.98	7.42
Pro^{3}	3.37	0.05/1.49	0.39/1.08	2.71/3.02								
Phe	4.32	3.08/3.27			7.22	7.28	7.21					
Gly	4.09/4.21											
Pro^{6}	4.35	2.03/2.43	2.11[b]	3.62/3.72								
Glu	4.45	2.00/2.36	2.44[b]									
Ala^{2}												
Leu	4.45	1.62/1.87	1.75	0.99/1.09								
Ala	4.41	1.34										
Pro^{3}	4.51	0.77/1.60	1.91/2.28	2.84/3.24								
Phe	4.46	3.13/3.36			7.27	7.34	7.28					
Gly	4.11/4.24											
Pro^{6}	4.27	1.97/2.38	2.08[b]	3.62/3.71								
Glu	4.40	1.99/2.27	2.44[b]									
Ala^{3}												
Leu	4.34	1.59[b]	1.65	0.85/1.09								
Trp	4.33	3.29/3.37						7.16	7.37	7.13	7.06	7.60
Ala	3.85	1.18										
Phe	4.53	3.02/3.21			7.29	7.30	7.23					
Gly	3.93/4.27											
Pro^{6}	4.32	2.00/2.32	2.06[b]	3.59/3.72								
Glu	4.31	1.98/2.21	2.38[b]									
Ala^{4}												
Leu	4.44	1.48/1.69	1.56	0.91/0.99								
Trp	4.56	3.24[b]						7.15	7.36	7.11	6.98	7.43
Pro^{3}	3.55	0.24/1.68	1.32/1.37	3.28[b]								
Ala	4.13	1.44										
Gly	4.01/4.10											
Pro^{6}	4.29	1.96/2.38	2.06[b]	3.56/3.65								
Glu	4.40	1.94/2.26	2.40[b]									
Ala^{5}												
Leu	4.62	1.40/1.92	1.77	1.01/1.08								
Trp	4.42	3.23/3.29						7.17	7.36	7.11	6.99	7.43
Pro^{3}	3.34	0.06/1.42	0.34/1.05	2.72/3.00								
Phe	4.27	3.02/3.31			7.21	7.31	7.23					
Ala	4.76	1.48										
Pro^{6}	4.35	1.93/2.47	2.09/2.23	3.81/3.87								
Glu	4.43	2.11/2.32	2.42[b]									
Ala^{6}												
Leu	4.58	1.60/1.88	1.77	1.01/1.12								
Trp	4.55	3.25/3.29						7.18	7.36	7.11	6.98	7.43
Pro^{3}	3.40	−0.04/1.47	0.39/1.05	2.63/2.97								
Phe	4.31	3.06/3.27			7.28	7.21	7.19					
Gly	3.96/4.19											
Ala	4.10	1.48										
Glu	4.43	2.03/2.29	2.45[b]									

(*continued overleaf*)

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Table 3 (*Continued*)

	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	$C^{o}H$	$C^{m}H$	$C^{p}H$	$C^{2}H$	$C^{4}H$	$C^{5}H$	$C^{6}H$	$C^{7}H$
Ala^{7}												
Leu	4.46	1.48/1.81	1.68									
Trp	4.42	3.19[b]						7.12	7.31	7.07	6.95	7.38
Pro^{3}	3.49	0.24/1.61	0.43/1.11	2.67/3.00								
Phe	4.31	3.03/3.26			7.16	7.24	7.15					
Gly	3.87/4.09											
Pro^{6}	4.20	1.95/2.34	2.00/2.04	3.41/3.61								
Ala	4.41	1.40										

[a] 600 MHz, CD_3OD; δ in ppm.
[b] 2H.

Table 4 ^{13}C[a]-NMR Data for the Ala-scan *Major* Analogues

	C^{α}	C^{β}	C^{γ}	C^{δ}	C^{o}	C^{m}	C^{p}	C^{2}	C^{4}	C^{5}	C^{6}	C^{7}
Ala^{1}												
Ala	49.36	15.42										
Trp	55.17	26.89						124.31	111.65	122.02	119.24	118.25
Pro^{3}	60.76	29.17	20.55	46.29								
Phe	57.28	36.77			129.09	128.98	127.59					
Gly	42.52											
Pro^{6}	62.36	29.49	24.78	46.43								
Glu	53.42	25.59	30.18									
Ala^{2}												
Leu	53.31	40.78	25.77	21.63/23.79								
Ala	49.96	15.22										
Pro^{3}	61.86	21.71	31.28	47.07								
Phe	57.82	37.74			129.69	129.73	128.15					
Gly	43.3											
Pro^{6}	63.18	30.33	25.43	47.18								
Glu	54.34	26.76	31.17									
Ala^{3}												
Leu	54.06	41.95	25.55	22.62/22.63								
Trp	57.43	27.36						124.91	112.06	122.57	119.84	119.25
Ala	51.92	16.70										
Phe	57.61	38.49			130.38	129.52	127.69					
Gly	43.13											
Pro^{6}	63.11	30.71	25.69	47.81								
Glu	54.01	26.63	31.45									
Ala^{4}												
Leu	53.32	40.92	25.98	22.24/23.79								
Trp	55.90	28.06						124.90	112.22	122.74	119.93	119.13
Pro^{3}	61.94	30.39	22.19	47.18								
Ala	52.52	17.13										
Gly	43.57											
Pro^{6}	63.34	30.52	25.64	47.35								
Glu	54.08	26.88	31.14									

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Table 4 (*Continued*)

	C^{α}	C^{β}	C^{γ}	C^{δ}	C^{o}	C^{m}	C^{p}	C^{2}	C^{4}	C^{5}	C^{6}	C^{7}
Ala^{5}												
Leu	53.29	41.78	26.11	22.57/23.88								
Trp	56.70	27.86						125.14	112.31	119.86	122.70	119.27
Pro^{3}	61.86	30.85	21.51	47.24								
Phe	58.24	38.41			129.82	129.82	127.90					
Ala	48.90	17.95										
Pro^{6}	64.80	30.15	26.73	48.44								
Glu	54.72	26.08	31.48									
Ala^{6}												
Leu	53.12	41.19	25.91	21.97/23.95								
Trp	55.87	27.94						124.78	112.34	122.64	119.83	119.06
Pro^{3}	61.61	29.93	21.60	47.03								
Phe	57.78	37.64			129.45	127.63	129.65					
Gly	42.96											
Ala	53.32	16.91										
Glu	54.38	26.90	31.23									
Ala^{7}												
Leu	52.16	39.93	24.96	21.49/23.44								
Trp	54.66	26.89						123.79	111.39	121.80	119.06	118.26
Pro^{3}	60.62	29.67	20.84	46.40								
Phe	56.90	36.84			126.76	128.63	128.75					
Gly	42.67											
Pro^{6}	62.30	29.72	24.97	46.33								
Ala	49.62	16.85										

[a] 150 MHz, CD_3OD; δ in ppm.

in DMF. Finally, the crude cyclopeptides, obtained by cleavage from the resin with 95% TFA, were purified by HPLC and analysed by ESMS and NMR spectrometry as reported in Tables 2–4.

The synthesis of the Ala^{7} analogue of hymenamide C (**8**) required a different synthetic strategy since the replacement of Glu with Ala did not permit the side-chain anchorage of the peptide to the solid support. The Fmoc/Boc strategy was used with the 2-chlorotritylchloride resin, which was loaded with Fmoc-Ala-OH (substitution degree: 0.5 mmol/g). The first amino acid was anchored to the linker by DIEA addition under anhydrous conditions, followed by capping of unreacted trityl groups with methanol. The resulting substitution degree was determined by UV spectrophotometric analysis (procedure **d**). The resin was then submitted to six coupling deprotection cycles to build the linear heptapeptide using the same conditions as those exploited for compounds **2–7**. When the linear heptapeptide was obtained, the Fmoc protecting group was removed from the *N*-terminal Leu residue and the N^{in}-Boc protected peptide was cleaved from the resin by using an AcOH/TFE/DCM solvent mixture. After HPLC purification the cyclization reaction was allowed to proceed in solution using HATU and DIEA in DCM; finally, after removing the Boc protecting group with 95% TFA, the purification on RP-HPLC yielded, as the major isomer, the Ala^{7} hymenamide C analogue with *cis* geometry at the Trp-Pro^{3} bond and *trans* geometry at the Gly-Pro^{6} bond, along with a lower amount of its *cis*^{3}-*cis*^{6} isomer.

Isomeric *cis* and *trans* Xxx-Pro forms can be distinguished in solution by careful analysis of the pattern of dipolar couplings in the ROESY spectra [12]. This ^{1}H NMR method relies on the observation that *cis* Xxx-Pro bonds are likely to give Hα-X/Hα-Pro NOE effects with the preceding residue in the sequence (Xxx), whereas an Hα-X/$H_2\delta$-Pro NOE effect is observed for *trans* Xxx-Pro bonds.

As expected, both Ala^{3} (**4**) and Ala^{6} (**7**) analogues, in which one of the Pro residues was replaced by Ala, yielded a single isomer. In particular, in the Ala^{3} analogue (**4**) NMR data suggested a *trans* geometry for the Gly-Pro^{6} linkage (ROESY cross-peak Hα-Gly/$H_2\delta$-Pro^{6}), while in the Ala^{6} analogue

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(**7**) a *cis* geometry for the Trp-Pro[3] amide linkage was indicated by the ROESY cross-peak Hα-Trp/Hα-Pro[3]. Quite surprisingly, when Trp and Phe were replaced (Ala[2] and Ala[4] analogues respectively), only one conformer was observed with *cis* geometry at the Trp-Pro[3] bond and *trans* geometry at the Gly-Pro[6] bond, as indicated by the ROESY cross-peak patterns. This result may be rationalized by the fact that freezing of the Pro[3] residue in the *cis* conformation would be associated with clustering of the aromatic amino acids located at positions $i-1$ and $i+1$ in the segment -Trp-Pro[3]-Phe- with the Pro pyrrolidine ring, or to the presence of a single aromatic residue either at position $i-1$ or $i+1$ [13]. On the contrary, in the Gly-Pro[6] segment, the Xxx-Pro bond accommodates in the more stable *trans* geometry.

Biological Activity

The biological activity of the seven modified cyclopeptides has been evaluated *in vitro* in two pharmacological models: they were tested for their ability to inhibit elastase degranulation release and to produce superoxide ions on human neutrophils (Table 5), while their capacity to reduce PGE_2 and NO levels was investigated on LPS stimulated macrophages (Table 6).

Overall, no dramatic changes were observed in the bioactivity for any Ala modified cyclopeptide. On

Table 5 Effect of Hymenamide C and Its Ala-Modified Analogues on Human Neutrophil Functions[a]

		Elastase degranulation release		Chemiluminescence	Viability
		%I (10 μM)	IC_{50}(μM)	%I (10 μM)	%I (10 μM)
1	Hymenamide C	54.2 ± 2.7^c	18.0	19.1 ± 2.0^b	0.0 ± 0.0
2	Ala[1] analogue	23.1 ± 2.4^c	—	8.2 ± 2.0^b	3.9 ± 1.3
3	Ala[2] analogue	43.1 ± 1.2^c	—	9.2 ± 2.1	2.1 ± 1.1
4	Ala[3] analogue	73.2 ± 3.1^c	8.3	—	2.2 ± 0.8
5	Ala[4] analogue	35.8 ± 4.0^c	—	0.0 ± 0.0	0.7 ± 0.7
6	Ala[5] analogue	51.4 ± 1.8^c	6.5	6.5 ± 2.8	0.0 ± 0.0
7	Ala[6] analogue	37.0 ± 3.5^c	—	1.9 ± 1.3	1.5 ± 1.0
8	Ala[7] analogue	58.8 ± 2.9^c	7.6	9.6 ± 3.2	1.1 ± 0.7

[a] Results show percentages of inhibition at 10 μM. The IC_{50}(μM) values were determined only for those compounds that reach 50% inhibition. Mean ± SEM ($n = 6$).
[b] $p < 0.05$.
[c] $p < 0.01$.

Table 6 PGE_2 and Nitrite Production in LPS-Stimulated Macrophages[a]

		PGE_2	Nitrite	Viability
		%I (10 μM)	%I (10 μM)	%I (10 μM)
1	Hymenamide C	41.8 ± 4.7^b	39.8 ± 2.9^b	0.4 ± 0.4
2	Ala[1] analogue	39.6 ± 4.9^b	9.3 ± 2.6	0.4 ± 0.4
3	Ala[2] analogue	37.9 ± 4.7^b	13.3 ± 3.3	0.0 ± 0.0
4	Ala[3] analogue	30.1 ± 4.8^b	9.8 ± 2.9	7.2 ± 2.4
5	Ala[4] analogue	36.1 ± 4.4^b	0.0 ± 0.0	9.6 ± 2.1
6	Ala[5] analogue	38.1 ± 4.9^b	12.9 ± 3.8	1.6 ± 1.5
7	Ala[6] analogue	38.3 ± 4.5^b	8.9 ± 3.1	2.6 ± 2.6
8	Ala[7] analogue	8.7 ± 2.8^b	13.4 ± 3.1	2.3 ± 0.7

[a] Results show percentages of inhibition at 10 μM. Mean ± SEM ($n = 6$).
[b] $p < 0.05$.

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the other hand, a more detailed inspection of the bioactivity profiles of these cyclopeptides revealed differences that were certainly above experimental error, thus allowing some useful conclusions to be derived on their structure–activity relationships. For instance the *ca.* 50% decrease shown in the elastase assay by the Ala^1 analogue ($Ile^1 \rightarrow Ala^1$) suggests that a hydrophobic side chain is preferred at this position. Even more interesting was the bioactivity increase found in the same assay for the Ala^3 derivative ($Pro^3 \rightarrow Ala^3$) which would indicate that the Trp-Pro^3 *cis* geometry is not a strict requirement. Another piece of information can be extracted from the small variation of the pharmacological profile caused by the $Glu^7 \rightarrow Ala^7$ substitution, thus showing that elimination of the negative charge at this level does not produce a relevant effect.

In other words, regarding PGE_2 production in macrophages and elastase release inhibition in neutrophils, all sequence positions of the cyclopeptide seem to tolerate well the presence of an Ala residue. Nevertheless, a pattern of structure–activity relationships emerged upon a more subtle analysis of the biological data. An exception to this trend was observed in the nitrite levels of LPS-stimulated macrophages where substitution with an Ala residue caused a marked decrease of activity in all cases.

In any case, these bioactivity results do appear rather unexpected if one takes into account the presence in the hymenamide C sequence of at least four side chains, namely those of Glu, Trp, Phe and Leu residues, capable of conveying specific interactions in a molecular recognition process.

As a preliminary conclusion, on the basis of the above data and considerations a non-receptorial mode of action cannot be excluded for these compounds.

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