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Synthesis, structural aspects and bioactivity of the marine cyclopeptide hymenamide C

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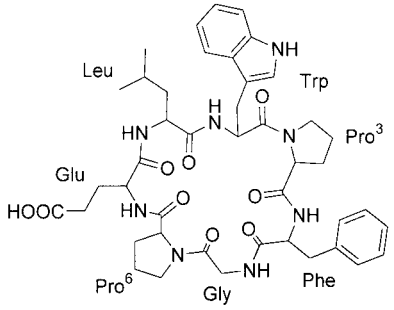
Abstract—Head-to-tail proline containing cyclopeptide hymenamide C [cyclo(Leu-Trp-Pro^3-Phe-Gly-Pro^6-Glu); **1**], isolated from a marine sponge and for which a preliminary immunomodulating activity was reported, was efficiently synthesized by a three-dimensional orthogonal solid-phase strategy (Fmoc/tBu/Allyl) via anchoring the ω-carboxyl function of the glutamic acid to the solid support (PAC–PEG–PS). The linear precursor was entirely assembled and subsequently cyclized on resin, yielding a major product identical to the natural hymenamide C and a minor one, a geometric isomer of hymenamide C (**2**), differing for the geometry of peptide linkages at Pro residues. Both the 'proline-rich' cyclopeptides were submitted to a multiparametric in vitro screening on immune cells in order to acquire additional information on their biological activity. Indeed, compounds **1** and **2** were shown to exert inhibitory effect on human neutrophil elastase degranulation release at micromolar concentration.

1. Introduction

In our systematic investigations of marine invertebrates as sources of structurally unique and potentially useful drugs,[1] we had the opportunity to investigate specimens of the marine sponge *Axinella carteri*[2] collected off the Vanuatu Islands (South Pacific), whose ethanolic extracts showed a preliminary immunomodulating activity. From this organism we isolated hymenamide C[3] (**1**) along with axinellins A and B and other cytotoxic cyclopeptides, such as axinastatins and stylostatins. These metabolites, characterized by an unusual high content of proline residues, belong to a rather large class of homodetic cyclopeptides with interesting bioactivities and remarkable structural similarity. In fact, these molecules, often referred to as 'proline rich',[4–8] are all hepta- or octa-cyclopeptides showing analogies in their amino acid content (many of them having an array of apolar residues with one or two aromatic residues) and, to some extent, also in their sequence. Intrigued by the variety of bioactivities reported for many members of this family of compounds and by the immunomodulating activity reported for hymenamide C[1,9] (**1**), we envisaged the need to produce by synthesis this compound in larger amounts, in order to be able to investigate both its structural properties and biopharmacological activities.



1 Hymenamide C (*cis*-Pro^3, *trans*-Pro^6; natural)

2 iso-Hymenamide C (*cis*-Pro^3, *cis*-Pro^6; synthetic isomer)

2. Results and discussion

The cyclic oligomeric structure of hymenamide C (**1**) led us to adopt a new solid-phase synthetic strategy based on a three-dimensional orthogonal scheme of protection which enables cyclization of the linear precursor still attached to the solid support via the side-chain of a tri-functional residue.[10] This approach for synthesizing cyclic peptides containing aspartic or glutamic acid derivatives, as well as other tri-functional amino acids, offers many advantages. These include: (a) the anchorage of the first (C-terminal) residue to the resin through the side-chain γ-carboxyl (carboxamide) function; (b) stepwise elongation of the linear sequence up to the desired length; (c) on resin cyclization by selective deprotection of the C-terminal residue

Keywords: cyclopeptides; solid phase synthesis; marine natural product; elastase degranulation.

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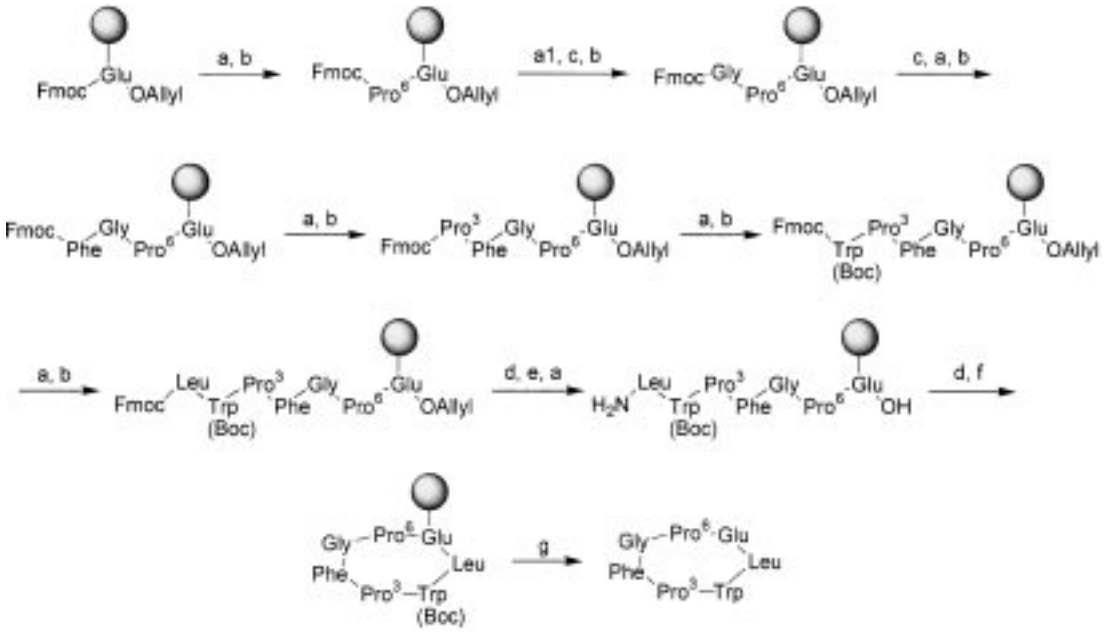

Figure 1. Solid-phase synthetic scheme of hymenamide C. a–d=see experimental: general procedures; e=$Pd^0(PPh_3)_4$ (4 equiv.), $CHCl_3$–AcOH–NMM (37:2:1), rt, under Ar; f=HOBt (4 equiv.), HBTU (4 equiv.), NMM (8 equiv.), DMF, rt, 5.5 h; g=TFA/H_2O (95:5) (10 μl×1 mg of resin), 1 h.

followed by intramolecular coupling with the free amino group of the N-terminal residue. In this way, the last crucial step can benefit from the pseudodilution effect attainable by controlling the degree of loading of the peptide on the resin. In our case, the first *N*-Fmoc-*O*-allyl protected glutamic acid anchored by side-chain to the solid support was *N*-deprotected using the general procedure employing piperidine in DMF (Fig. 1). The resin was subsequently submitted to six coupling-deprotection cycles to build the linear heptapeptide. Each amino acid coupling step was monitored by the ninhydrin test, while the formation of peptide bonds was accomplished using HBTU [*O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate] as activating agent in the presence of HOBt (1-hydroxybenzotriazole) and NMM (*N*-methyl-morpholine) in DMF.

Table 1. ^1H and ^{13}C NMR data for 1 (600 MHz, CD_3OD)

Position	^1H[a]	^{13}C[a]	Position	^1H[a]	^{13}C[a]
Trp			Gly		
α	4.53	54.84	α	4.09	42.26
β	3.25[b]	26.81		4.24	
1					
2			Pro^3		
3			α	4.34	62.52
4	7.35	111.02	β	2.02	29.89
5	7.10	121.62		2.44	
6	6.97	118.84	γ	2.11[b]	24.50
7	7.41	117.82	δ	3.63	46.19
				3.72	
Pro^3					
α	3.42	60.84	Glu		
β	0.01	29.02	α	4.45	53.46
	1.50		β	1.99	25.90
γ	0.40	20.22		2.29	
	1.07		γ	2.43[b]	30.31
δ	2.97	45.85			
	2.61		Leu		
			α	4.56	52.26
Phe			β	1.61	40.03
α	4.29	57.24		1.89	
β	3.07	36.53	γ	1.77	25.07
	3.27		CH_3	1.00	23.04
1			CH_3	1.13	20.83
2,6	7.19	128.56			
3,5	7.29	128.49			
4	7.23	126.58			

[a] δ in ppm.
[b] 2H.

To avoid the undesired DKP (diketopiperazine) formation at the dipeptide level during Fmoc (9-fluorenylmethoxycarbonyl) removal in piperidine, an unwanted side reaction that is reported to be promoted by the presence of the allyl ester at C-terminus[11] and the Pro^6 residue in the second position, we performed a fast Fmoc deprotection cycle before the second coupling reaction (procedure a1, see Section 3). After introducing the third amino acid residue (Gly), we monitored the amount of DKP formation by performing a UV spectrometric analysis of the Fmoc-piperidine adduct chromophore by measuring the absorbance at λ_{max}=301 nm. In the latter conditions (but not in standard conditions), only a low amount of DKP was observed.

After the synthesis of the linear heptapeptide was accomplished, full deprotection was achieved by submitting the resin-bound peptide to treatment with $Pd[(PPh_3)_4]$ in $CHCl_3$–AcOH–NMM under an Ar atmosphere to remove the allyl protecting group at C-terminus, and subsequently the Fmoc protecting group was removed at the N-terminal Leu residue. Then, the cyclization reaction was allowed to proceed on solid-phase with HOBt–HBTU–NMM activation in DMF and finally the crude product (23 mg, 27% overall unoptimized yield based on a resin loading of 0.14 mmol g^{-1} as determined by UV analysis according to procedure c) was removed from the linker by using 95% TFA in water. This mixture was purified on RP-HPLC (on a Jupiter C18 column eluting with CH_3CN/H_2O gradient, each

Table 2. ^{1}H and ^{13}C NMR data for 2 (600 MHz, CD_3OD)

Position	$^{1}H^{a}$	$^{13}C^{a}$	Position	$^{1}H^{a}$	$^{13}C^{a}$
Trp			Gly		
α	4.40	55.93	α	4.08	43.30
β	3.27	27.84		4.13	
	3.21				
1					
2			Pro^6		
3			α	3.62	46.56
4	7.40	119.13	β	2.04	25.97
5	6.99	120.12		2.25	
6	7.12	122.59	γ	0.86	[c]
				1.63	
7	7.37	112.25	δ	1.47	[c]
				3.02	
Pro^3			Glu		
α	3.41	61.63	α	4.36	54.10
β	−0.03	30.03	β	2.08	29.74
	1.48			2.31	
γ	0.36	35.02	γ	2.48[b]	30.89
	1.02				
δ	2.68	46.99			
	2.97		Leu		
			α	4.67	52.08
Phe			β	1.32	40.99
α	4.31	58.36		1.86	
β	3.06	38.10	γ	1.79	24.72
	3.00		CH_3	0.96	23.54
1			CH_3	1.05	22.25
2,6	7.20	129.52			
3,5	7.29	129.50			
4	7.24	127.49			

[a] δ in ppm.
[b] 2H.
[c] These signals were not assigned because they were lost in the noise.

containing 0.1% TFA) yielding two products that were shown to be isomeric by ESMS and ^{1}H-NMR experiments. The major product was identical to the natural hymenamide C[3] (**1**), as its spectral properties were superimposable to those of the natural product (Table 1), while the minor component **2**, exhibiting a slightly different ^{1}H-NMR spectrum (Table 2), was identified as a geometric isomer of hymenamide C, differing by the *cis* geometry of the peptide linkage at the Pro^6 residue.

Isomeric *cis*- and *trans*-Pro forms can be distinguished in solution by careful analysis of the pattern of dipolar couplings in ROESY spectra.[12] This ^{1}H NMR method relies on the observation that *cis*-Pro residues are likely to give Hα-X/Hα-Pro NOE effects with the preceding residue in the sequence (X), whereas an Hα-X/H_2δ-Pro effect is observed for *trans*-Pro geometries. In our synthetic hymenamide C (**1**), NMR data suggested a *cis* geometry for the Trp-Pro^3 peptide bond (ROESY cross-peak Hα-Trp/Hα-Pro^3) and a *trans* geometry for the Gly-Pro^6 linkage (ROESY cross-peak Hα-Gly/H_2δ-Pro^6), in perfect agreement with that reported for the natural product[3] (Fig. 2). NMR data of the hymenamide C isomer (**2**) pointed to the same *cis* geometry for the Trp-Pro^3 peptide bond (ROESY cross-peak Hα-Trp/Hα-Pro^3), while the geometry of the Gly-Pro^6 linkage was established to be *cis* (ROESY cross-peak Hα-Gly/Hα-Pro^6) (Fig. 2).

In addition, we made a comparison of some structural aspects of the two cyclopeptides. Temperature coefficient experiments on hymenamide C (**1**) ($\Delta\delta/\Delta T$=−0.5, −0.7, −0.6, −3.1, −6.2 for NH of Phe, Gly, Leu, Glu and Trp, respectively) were consistent with those reported for the natural compound and establishing that the amide proton of Trp was oriented outside the peptide ring, while the three amide protons of Phe, Gly and Leu residues participated in intramolecular hydrogen bonds, composing a β bulge motif.[3] This type of non-repetitive structure is defined as a region between two consecutive β-type hydrogen bonds which include two residues on one strand opposite a single residue on the other strand.[13] In hymenamide C (**1**) the two consecutive Phe and Gly residues on one strand are involved in hydrogen bonds with the Leu residue on the opposite strand (NH-Phe/CO-Leu; NH-Gly/CO-Leu; NH-Leu/CO-Gly).[3] Moreover, the analysis of ROESY cross-peaks pattern confirmed the existence of two types of β turn in **1**: a type II[14] at Pro^6-Glu level and a type VI(a)[15] at the linkage Trp-Pro^3, implying that hymenamide C (**1**) exists as single predominant conformation. In detail: type II β turn, which is common for a *trans*-Pro residue, was suggested at Pro^6-Glu level by the observation of strong ROESY cross-peaks between Hα-Pro^6/NH-Glu; NH-Glu/NH-Leu ($i/i+1$; $i+1/i+2$). Furthermore, the type VI(a) β turn was supported at Trp-Pro^3 level by the presence in the ROESY spectrum of strong correlation peaks between Hα-Trp/NH-Phe and Hα-Trp/NH-Gly ($i/i+2$; $i/i+3$). The minor synthetic hymenamide C isomer **2** showed a somewhat different pattern of NH amide proton temperature coefficients ($\Delta\delta/\Delta T$). In particular, Trp $\Delta\delta/\Delta T$ value was close to that measured for the same residue in **1**, while Phe, Gly and Leu residues yielded $\Delta\delta/\Delta T$ coefficients of intermediate value, suggesting that multiple conformational equilibria may be involved in the case of **2** ($\Delta\delta/\Delta T$=−2.3, −2.17, −3.7, −7.2, −7.2 for NH of Phe, Gly, Leu, Glu and

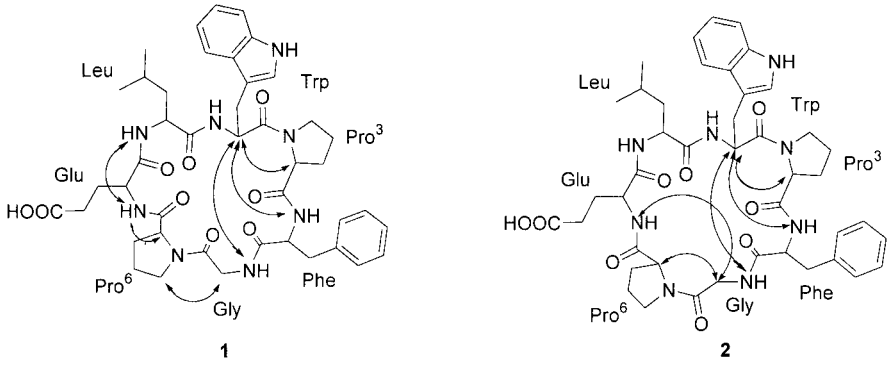

Figure 2. Key ROESY correlations for hymenamide C (**1**) and *iso*-hymenamide C (**2**).

Table 3. Effect of hymenamides on human neutrophil functions

Compound	Elastase degranulation release		Chemiluminescence	Viability
	%I (10 μM)	IC_{50} (μM)	%I (10 μM)	%I (10 μM)
1	54.2±2.7[a]	18.0	19.1±2.0[a]	0.0±0.0
2	44.9±5.2[a]	–	15.2±4.1	0.0±0.0

Results show percentages of inhibition at 10 μM and IC_{50} (μM) values determined only for those compounds that reach 50% of inhibition. Mean±S.E.M. (n=6).
[a] $p<0.01$.

Trp, respectively). The pattern of ROESY cross-peaks was consistent with the hypothesis of a greater conformational mobility of **2**: in fact, dipolar contacts across the Trp-Pro[3]-Phe-Gly segment were almost identical in **1** and **2**, while in **2** an unambigously determined secondary structure (type VI(a) β turn) for the Gly-Pro[6]-Glu-Leu sequence could not be inferred from ROESY data, despite the *cis*-geometry of the Gly-Pro[6] linkage (see Fig. 2).

Our bioactivity screening, prompted by the initial report of immunomodulating properties of hymenamide C[1,9] (**1**), moved in the direction of shedding more light on the biological effects of this molecule through a multiparametric analysis of immune cell functions, such as neutrophil elastase degranulation and production in macrophages of pro-inflammatory mediators like PGE_2 and NO (Tables 3 and 4). In detail, the most relevant effect of hymenamide C (**1**) is in our opinion its ability to cause a 54.2% inhibition of the elastase degranulation release at the concentration of 10 μM (IC_{50}=18.0 μM), while the isomeric *cis–cis* form caused about 44.9% inhibition (Table 3). These results are very interesting as neutrophils are recruited into the inflammatory sites in a great variety of chronic diseases. It is known that inhibitors of human neutrophil elastase degranulation may exert potent therapeutic effects on pulmonary emphysema,[16] adult respiratory distress syndrome,[17] and other diseases involving tissue degradation.[18–20] We wish to note that cyclosporine, the cyclopeptidic standard reference drug for immunosuppressive activity, exerts a weaker inhibitory effect on elastase degranulation release in the same assay (28.2% of inhibition at the conc. of 100 μM). On the other hand, we must consider that hymenamide C has no ability to interfere with lymphocyte proliferation, whereas cyclosporine does so at nanomolar levels, thus implying that hymenamides may display effects that in a way complement those of standard immunosuppressors. In addition, hymenamide C (**1**) showed a 41.8% inhibition of PGE_2 production and a 39.8% inhibition of nitrite production in 18h LPS-stimulated macrophages (RAW 264.7) at the same 10 μM concentration (Table 4). The unnatural isomeric *cis–cis* form of hymenamide C (**2**) was about 4 and 1.5 times less active, respectively, than the natural product.

As concerning structure–activity relationships, the present data suggest that several additional derivatives, related to both hymenamides (**1** and **2**), are needed in order to draw preliminary conclusions as, to the best of our knowledge, such compounds are the first among proline-rich cyclopeptides for which interference with immune cells has been reported. Ongoing studies, aimed at the synthesis and biological evaluation of peptide analogues, designed following an *Ala*-scan[21] approach, are currently underway in our laboratories and will be reported in due course.

3. Experimental

3.1. General methods

Unless specified, solvents were reagent grade; they were purchased from Aldrich or Fluka or LabScan 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 MeCN were HPLC grade. Fmoc-Glu(*O*-PAC–PEG–PS)-*O*-allyl [the Fmoc-Glu-*O*-allyl already linked by side-chain to *p*-alkoxybenzyl alcohol (PAC) polyethylene glycol–polystyrene (PS) support] was purchased from Perseptive Biosystems (loading capacity 0.18 mmol g^{-1}). The Fmoc-L-amino acids and the coupling reagents (HOBt and HBTU) were supplied by Novabiochem and used without further purification. Solid-phase reactions were carried out in batch, on a glass reaction vessel and using the Fmoc/*t*Bu/Allyl protocol. For quantification of Fmoc amino acid on the resin, absorbance at 301 nm was read employing a Shimadzu UV 2101 PC. The ^1H and ^{13}C (^1H–^1H and ^1H–^{13}C) spectra were recorded using a Bruker DRX 600 MHz spectrometer with a deuterated solvent (CD_3OD or DMSO). The ^1H NMR temperature coefficients experiments were conducted at 27, 30, 35, 40, 45, 50, 55, and 60°C. LCQ Finnigan mass spectrometer was used to record the ESMS spectra.

3.2. General procedures for solid-phase reactions

(a) Fmoc deprotection: 20% piperidine in DMF (3 ml), 1.5 min; 3 ml of 20% piperidine in DMF, 10 min; washings

Table 4. PGE_2 and nitrite production in 18h LPS-stimulated macrophages (RAW 264.7)

Compound	PGE_2 %I (10 μM)	Nitrite %I (10 μM)	Viability %I (10 μM)
1	41.8±4.7[a]	39.8±2.9[a]	0.4±0.4
2	9.7±5.8	27.0±1.8[a]	0.0±0.0

Results show percentages of inhibition at 10 μM. Mean±S.E.M. (n=6).
[a] $*p<0.01$.

(1.5 min each): 2×3 ml of DMF, 3×3 ml of DCM, 2×3 ml of DMF. [a1] 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. (b) Peptide coupling conditions: HOBt (5 equiv.), HBTU (5 equiv.), Fmoc-amino acid (5 equiv.) NMM (6 equiv.) in DMF (341 μl/100 mg of resin), 2 h; washings (1.5 min each): 3×3 ml of DMF, 3×3 ml of DCM. (c) Spectrometric 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 rt in the sealed flasks. 1.6 ml of MeOH were 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 shaker and the absorbance of the samples versus the reference solution was measured at 301 nm. The substitution level (in mmol of amino acid/g of resin) was calculated from the equation: mmol $g^{-1}=(A_{301}/7800)\times$ (10 ml g^{-1} of resin). (d) Minicleavage 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 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 falcon 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 ether, suspended in HPLC water and, after lyophilization, analyzed by RP-HPLC to determine the success of the reaction by the relative intensity of the peaks assigned to protected and deprotected peptide.

3.3. Hymenamide C synthesis

Fmoc-Glu(*O*-PAC–PEG–PS)-*O*-allyl (733.4 mg, 0.14 mmol g^{-1} loading level) was placed into a 50 ml reaction glass vessel and allowed to swell for 45 min with 3 ml of DMF. The first protected Fmoc-amino acid, already anchored by side-chain on the PAC–PEG–PS resin, was Fmoc deprotected according to general procedure a. The resin was subsequently submitted to the following series of six coupling-deprotection cycles: (i) peptide coupling according to general procedure b with N^{α}-Fmoc-L-proline (178.1 mg, 0.528 mmol), HOBt (80.8 mg, 0.528 mmol), HBTU (200.2 mg, 0.528 mmol) and NMM (72.56 μl, 0.660 mmol), followed by Fmoc deprotection according to general procedure a1 to avoid the formation of DKPs. The result of the Fmoc removal was monitored according to general procedure c; (ii) peptide coupling according to general procedure b with N^{α}-Fmoc-L-glycine (156.9 mg, 0.528 mmol), HOBt (80.8 mg, 0.528 mmol), HBTU (200.2 mg, 0.528 mmol) and NMM (72.56 μl, 0.660 mmol), followed by determination, according to general procedure c, of the level of substitution of the resin bound peptide. As the DKP formation was less than 15%, the Fmoc deprotection was performed according to general procedure a; (iii) peptide coupling according to general procedure b with N^{α}-Fmoc-L-phenylalanine (204.5 mg, 0.528 mmol), HOBt (80.8 mg, 0.528 mmol), HBTU (200.2 mg, 0.528 mmol) and NMM (72.56 μl, 0.660 mmol), followed by Fmoc deprotection according to general procedure a; (iv) peptide coupling according to general procedure b with N^{α}-Fmoc-L-proline (178.1 mg, 0.528 mmol), HOBt (80.8 mg, 0.528 mmol), HBTU (200.2 mg, 0.528 mmol) and NMM (72.56 μl, 0.660 mmol), followed by Fmoc deprotection according to general procedure a; (v) peptide coupling according to general procedure b with N^{α}-Fmoc-L-tryptophan(Boc) (278 mg, 0.528 mmol), HOBt (80.8 mg, 0.528 mmol), HBTU (200.2 mg, 0.528 mmol) and NMM (72.56 μl, 0.660 mmol), followed by Fmoc deprotection according to general procedure a; (vi) peptide coupling according to general procedure b with N^{α}-Fmoc-L-leucine (186.6 mg, 0.528 mmol), HOBt (80.8 mg, 0.528 mmol), HBTU (200.2 mg, 0.528 mmol) and NMM (72.56 μl, 0.660 mmol).

The ninhydrin test was performed after each amino acid coupling step and the coupling repeated if necessary. A small sample of the resin bound peptide was analyzed according to general procedure d to determine the retention time of the fully protected peptide. 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 equiv.) in $CHCl_3$–AcOH–NMM (37:2:1) (mechanical shaking overnight at rt and under Ar) to remove the allyl protecting group of the C-terminus of the peptide anchored to the resin. The fully deprotected linear anchored heptapeptide was obtained by washing the resin with 70 ml of DIEA–DMF (0.5:99.5), 70 ml of sodium diethyldithiocarbamate in DMF (0.5:99.5 w/w) and subsequently by removing the Fmoc protecting group of the N-terminus of the leucine (20% piperidine in DMF). A sample of resin bound peptide was collected after each deprotection step and submitted to minicleavage according to general procedure d to determine the progress of the deprotection reaction. Cyclization was then performed on solid-phase by 4 equiv. of HOBt, 4 equiv. of HBTU and 8 equiv. 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. The support-bound peptide was washed with DMF and DCM and then dried. The resin bound peptide was deprotected from Boc (*t*-butoxycarbonyl group) and cleaved from the solid support by treatment with 10 μl×1 mg of resin (8 ml) of TFA/H_2O (95:5) for 1 h, under stirring. The cleavage mixture was filtered off and the resin was washed three times with TFA 95% (6 ml). The filtrate volume was reduced under a nitrogen stream and then added with a twenty-fold excess of cold diethyl ether. A white precipitate appeared soon after ether addition. For maximum recovery, the ether peptide mix 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/Gelman Laboratory) installed on a glass 47 mm filter holder with a vacuum aspirator. The precipitate was washed with cold ether. The filter was transferred to a glass vessel, the solid removed by HPLC-grade water (some drops of glacial acetic acid) and ultrasounds, and the suspension was lyophilized. The crude cyclopeptide was analyzed by RP-HPLC on a Jupiter C-18 analytical column (250×4.60 mm, 5 μ, 300 Å), using a 40 min

gradient of 25–50% CH_3CN/H_2O (each containing 0.1% TFA) at a flow rate of 1.0 ml min^{-1} and detecting at 210 nm. The HPLC showed two main peaks of different intensity: the major one was identified as synthetic hymenamide C (**1**) by coinjection with the natural sample, while the minor one was identified as a geometric isomer of hymenamide C (**2**) on the basis of ESMS and ^{1}H-NMR experiments. The crude cyclopeptides were then purified by semipreparative RP-HPLC on a Jupiter C-18 column (250×10.00 mm, 10 μ, 300 Å), using the same elution conditions of the analytical HPLC at a flow rate of 5.0 ml min^{-1}, with UV detector at λ=300 nm. The HPLC yielded successively, as white solids, *cis–trans* hymenamide C (**1**) (3.6 mg, R_t= 32.31 min; ESMS, *m/z* 827 for $[M+H]^+$) and *cis–cis* iso-hymenamide C (**2**) (2.2 mg, R_t=30.96 min; ESMS, *m/z* 827 for $[M+H]^+$; HRESIMS, *m/z* 827.4043 for $[M+H]^+$).

3.4. 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 rt. Contaminating erythrocytes were lysed by hypotonic treatment using ice-cold distilled water for 20 s. The cell pellets were gently resuspended in 10 ml ice-cold modified phosphate buffer saline free of Ca^{2+} and Mg^{2+}, 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 rt. Neutrophils were separated and resuspended in phosphate buffer saline[22] containing 1.26 mM Ca^{2+} and 0.9 mM Mg^{2+}. Viability was greater than 95% by the trypan blue exclusion test.

3.5. Cell viability assays

The mitochondrial dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan[23] was used to assess the possible cytotoxic effect of hymenamide compounds on human neutrophils as well as on the mouse macrophage cell line RAW 264.7.

3.6. Elastase release by human neutrophils

Aliquots of 1.0 ml human neutrophils (2.5×10^6cells ml^{-1}) were preincubated at 37°C for 5 min with 10 μl of hymenamide compounds dissolved in ethanol (or an equivalent volume of ethanol for the controls). After this, the tubes were stimulated for a further 10 min at 37°C using as stimuli: cytochalasin B (10 μM) and *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP, 10 nM). Elastase activity was estimated in aliquots of stimulated human neutrophils supernatants incubated with *N-tert*-butoxy-carbonyl-L-alanine *p*-nitrophenyl ester. The extent of *p*-nitrophenol release was measured at 414 nm in a microplate reader. The direct effects on elastase were also tested using aliquots of supernatants of cytochalasin B+fMLP-stimulated human neutrophils.[24]

3.7. Chemiluminescence

The chemiluminescence generated by human neutrophils stimulated with TPA (1 μM) in the presence of luminol (40 μM) was recorded in a Microbeta Trilux counter (Wallac., Turku, Finland).[25]

3.8. Cell culture

The mouse macrophage cell line RAW 264.7 (European Collection of Cell Cultures) was cultured in Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine 2 mM, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 10% fetal bovine serum. Macrophages were removed from the tissue culture flask using a cell scraper, centrifuged at 800 *g* for 10 min. Cells were resuspended at a concentration of 2×10^6 ml^{-1} in a total volume of 200 μl and cultured in 96-well culture plate. Macrophages were co-incubated with hymenamide compounds and *Escherichia coli* LPS (serotype 0111:B4) (10 μg ml^{-1}) at 37°C for 18 h. Prostaglandin E_2 and nitrite production were determined in 18h supernatants.

3.9. Statistical analysis

The results are presented as means±S.E.M (Standard Error Media); *n* represents the number of experiments. Inhibitory concentration 50% (IC_{50}) values were calculated from at least four significant concentrations (*n*=6). The level of statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons.

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