Tetrahedron 65 (2009) 10424–10429

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Perthamides C and D, two new potent anti-inflammatory cyclopeptides from a Solomon Lithistid sponge *Theonella swinhoei*

Carmen Festa [a], Simona De Marino [a], Valentina Sepe [a], Maria Chiara Monti [b], Paolo Luciano [a], Maria Valeria D'Auria [a], Cecile Débitus [c], Mariarosaria Bucci [d], Valentina Vellecco [d], Angela Zampella [a,*]

[a] Dipartimento di Chimica delle Sostanze Naturali, Università di Napoli 'Federico II', via D. Montesano 49, 80131 Napoli, Italy
[b] Dipartimento di Scienze Farmaceutiche, Università di Salerno, via Ponte don Melillo, Fisciano (SA) 84084, Italy
[c] Institut de Recherche pour le Développement (IRD), Polynesian Rresearch Center on Island Biodiversity, BP529, 98713 Papeete, Tahiti, French Polynesia
[d] Dipartimento di Farmacologia Sperimentale Università di Napoli 'Federico II', via D. Montesano 49, 80131 Napoli, Italy

ARTICLE INFO

Article history:
Received 8 June 2009
Received in revised form
17 September 2009
Accepted 8 October 2009
Available online 13 October 2009

ABSTRACT

Two new metabolites, perthamides C and D, have been isolated from the marine sponge *Theonella swinhoei*. Their structures were determined by interpretation of NMR and ESIMS data. All compounds exhibited in vivo potent anti-inflammatory activity. Biological activity and structural elucidation are reported.

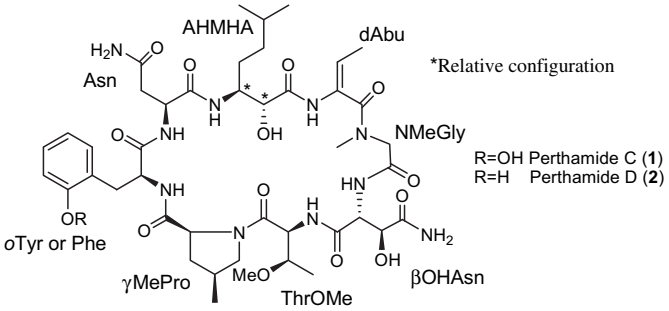
1. Introduction

Marine sponges of the genus Theonella are an excellent source of bioactive peptides with interesting biological activities. The diverse array of Theonella derived peptides range from acyclic peptides, such as polytheonamides,[1] koshikamides A1, and A2;[2,3] cyclic peptides, exemplified by cyclotheonamides,[4–6] kombamide,[7] orbiculamide,[8] barangamide,[9] keramamides,[10–14] cupolamide,[15] oriamide;[16] large-ring bicyclic peptides, such as theonellamides;[17] depsipeptides headed by theonellapeptolides,[18,19] cyclolithistide,[20] koshikamides A and B,[21] papuamides,[22] nagahamide;[23] theopapuamide[24] and glycopeptides.[25,26]

As for other peptides of marine origin, the most striking feature of these metabolites is the preponderance of non-ribosomial amino acids suggesting that these metabolites are the result of the NRPS/PKS pathways of microorganisms, suggesting that symbiotic microorganisms,[27–30] including cyanobacteria are the real producers of the sponge metabolites.

In the frame of a project directed to the chemical investigation and valorisation of the marine invertebrates of South Pacific we have the opportunity to study the sponge *Theonella swinhoei* collected at the Solomon Islands, whose crude extracts exhibited a moderate anti proliferative activity.

The purification of the polar extracts afforded two new cyclic peptides related to perthamide B, a cytotoxic peptide isolated from an Australian collection of a *T. swinhoei* sponge,[31] which we named perthamides C (**1**) and D (**2**). In this paper we describe the isolation, the structure elucidation, including the stereochemical characterisation and the biological activity of the new peptides.



2. Results and discussion

The lyophilised sponge (207 g) was extracted with MeOH, and the combined extracts were fractionated according to the Kupchan partitioning procedure.[32] The *n*-BuOH extract was purified by DCCC (*n*-BuOH/Me_2CO/H_2O, descending mode) followed by reverse-phase HPLC to give perthamides C (**1**) and D (**2**).

Perthamide C (**1**) was obtained as amorphous solid, which analysed for the molecular formula $C_{43}H_{64}N_{10}O_{14}$ by high-resolution ESIMS mass spectrometry coupled with ^1H and ^{13}C NMR spectral data (Table 1). The peptidic nature was evident from the presence of six exchangeable NH protons from δ_H 9.40–6.72 in the ^1H NMR and acyl carbons (δ_C 174.8–169.7) in the ^{13}C NMR spectra obtained in CD_3OH.

* Corresponding author. Tel.: +39 081678525; fax: +39 081678552.
E-mail address: azampell@unina.it (A. Zampella).

0040-4020/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tet.2009.10.026

Table 1
^{1}H and ^{13}C NMR data for perthamides C (**1**) and D (**2**) (CD_3OH, 700 MHz)

1				**2**			
Residue	δ_H[a]	δ_C	HMBC	Residue	δ_H[a]	δ_C	HMBC
ThrOMe				**ThrOMe**			
1	—	174.3		1	—	172.9	
2	5.16 br d (6.1)	57.7		2	5.03 ovl	57.6	1
3	4.26 ovl	74.8		3	4.34 m	75.0	2
4	1.36 d (6.1)	15.6	2, 3	4	1.36 d (6.1)	15.3	2, 3
OMe	3.42 s	56.0	3	OMe	3.42 s	56.0	3
NH	9.40 br s			NH	9.34 br s		
γMePro				**γMePro**			
1	—	174.3		1	—	173.3	
2	4.09 dd (11.3, 6.8)	65.4	3	2	4.19 dd (11.5, 5.4)	64.8	1
3	2.15 dt (11.3, 5.7)	37.8	4, 5	3	2.23 m,	37.7	
	0.91 m		1, 2, 4, 5, 6		0.79 m		1, 2, 4
4	2.32 m	35.4	3, 5, 6	4	2.29 m	35.3	
5	4.23 t (8.4)	55.3	2, 3, 4, ThrOMe-1	5	4.06 t (8.2)	55.4	4
	3.33 ovl				3.20 ovl		4, ThrOMe-1
6	1.14 d (6.3)	16.5	3, 4, 5	6	1.06 d (6.3)	16.0	3, 4, 5
***o*Tyr**				**Phe**			
1	—	173.9		1	—	173.2	
2	4.25 ovl	59.1	1, 3, 1′	2	4.64 m	55.9	1, 3, 1′
3	3.14 t (13.3)	32.3	1, 2, 1′, 2′, 6′	3	3.39 ovl	38.0	2, 1′, 2′
	2.95 dd (13.3, 3.1)				2.80 dd (11.5, 14.1)		1, 2, 1′, 2′
1′	—	125.6		1′	—	137.8	
2′	—	155.7		2′,6′	7.24 d (7.4)	129.6	3, 3′, 4′
3′	6.84 d (7.8)	116.1	1′, 2′, 5′	3′,5′	7.36 t (7.6)	129.7	2′
4′	7.14 t (7.8)	129.6	2′, 6′	4′	7.29 t (7.3)	127.9	
5′	6.87 t (7.8)	121.9	1′, 3′, 4′, 6′	NH	6.39 d (8.0)		1
6′	7.23 d (7.8)	132.1	3, 2′, 4′				
NH	7.56 d (6.1)		1, 2, 3, MePro-1				
Asn				**Asn**			
1	—	173.3		1	—	173.1	
2	4.96 ovl	50.0		2	4.90 ovl	50.1	
3	3.43 ovl	38.0	2, 4	3	3.36 ovl	37.8	2, 4
	2.58 dd (17.3, 2.7)		1, 2, 4		2.58 dd (17.7, 3.3)		1, 2, 4
4	—	174.8		4	—	174.7	
NH	7.46 d (10.2)		2, *o*Tyr-1	NH	7.37 d (4.4)		1
NH_2	7.72 br s	—	4	NH_2	7.73 s		4
	6.74 br s		3, 4		6.81 s		3, 4
AHMHA				**AHMHA**			
1	—	173.7		1	—	172.9	
2	4.43 br s (1.0)	74.4	1, 3, 4	2	4.40 s	74.3	1, 3
3	4.16 br dd (9.6, 1.0)	54.7		3	4.20 ovl	54.3	
4	1.59 m, 1.29 ovl	25.7	3, 5, 6	4	1.54 m, 1.32 ovl	25.6	
5	1.27 ovl, 1.03 m	36.0	3, 4, 6, 7, 8	5	1.27 ovl, 1.03 m	35.8	
6	1.48 m	28.8	4, 5, 7, 8	6	1.49 m	28.7	
7	0.82 d (6.1)	22.6	5, 6, 7	7	0.84 d (6.1)	22.6	5, 6, 7
8	0.81 d (6.1)	22.8	5, 6, 8	8	0.83 d (6.4)	22.8	5, 6, 8
NH	6.72 d (9.3)		3, 4, Asn-1	NH	6.68 d (9.0)		Asn-1
OH	5.20 br s			OH	n.o		
dAbu				**dAbu**			
1	—	170.8		1	—	170.6	
2	—	131.9		2	—	132.0	
3	5.96 q (6.9)	128.9	1	3	5.94 q (7.0)	128.3	4
4	1.68 d (6.9)	12.6	1, 2, 3	4	1.69 d (7.0)	12.5	1, 2, 3
NH	9.08 br s			NH	9.01 br s		
***N*MeGly**				***N*MeGly**			
1	—	169.8		1	—	169.2	
2	4.32 ovl,	53.7	1, *N*Me	2	4.18 ovl,	53.5	1, *N*Me
	3.70 d (17.6)				3.68 d (18.0)		1, dAbu-1
N-Me	2.92 s	36.0	2, dAbu-1	*N*-Me	2.93 s	35.6	2, dAbu-1
βOHAsn				**βOHAsn**			
1	—	169.7		1	—	169.6	
2	5.40 t (6.5)	54.8	1	2	5.35 t (6.6)	54.6	
3	4.94 ovl	77.8		3	4.82 ovl	77.5	1, 2, 4
4	—	172.2		4	—	171.7	
NH	7.81 br s			NH	n.o		
NH_2	9.40 d (6.5)			NH_2	9.32 d (6.6)		

Ovl: overlapped; n.o: not observed.
[a] Coupling constants are in parentheses and given in hertz. ^{1}H and ^{13}C assignments aided by COSY, TOCSY, HSQC and HMBC experiments.

Extensive analysis of the ^{1}H and ^{13}C NMR data of **1**, including ^{1}H–^{1}H COSY, HSQC, HMBC spectra (see Table 1), by comparison with those of perthamide B,[31] disclosed the presence of one residue each of asparagine (Asn), β-hydroxyasparagine (βOHAsn), *O*-methylthreonine (ThrOMe), 2-amino-2-butenoic acid (dAbu), *N*-methylglycine (*N*MeGly), γ-methylproline (γMePro). The NMR spectra also suggested the presence of a 2-substituted phenolic ring δ_H 6.84 (d, *J* 7.8), 7.14 (t, *J* 7.8), 6.87 (t, *J* 7.8), and 7.23 (d, *J* 7.8), that on the basis of HMBC data was assigned to an *o*-tyrosine subunit. *o*-Tyrosine is well know as product of the oxidation of phenylalanine residue in proteins and is reported to be an endogenous biomarker of oxidative damage.[33,34] To the best of our knowledge it is unprecedented in marine natural products.

The last spin system in perthamide C (**1**) was identified as 3-amino-2-hydroxy-6-methylheptanoic acid (AHMHA), which is also unprecedented in natural peptides. The complete spin system was inferred from COSY and TOCSY data. An acyl group was placed at C1 on the basis of HMBC cross peak between the hydroxymethine proton at C2 (δ_H 4.43) with a carbonyl at δ 173.7 ppm.

The complete sequence of **1** was secured from the inter-residue NOE NH/CHα and NCH$_3$/CHα interactions and HMBC correlations acquired in CD$_3$OH (Table 1 and Fig. 1).

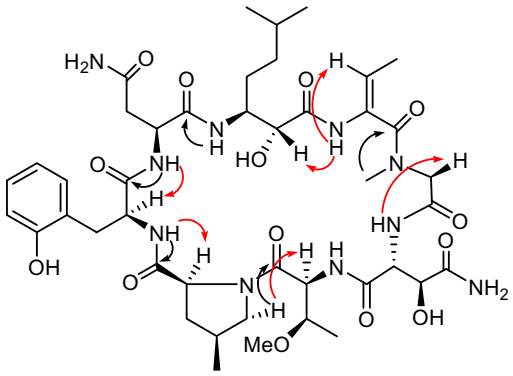


Figure 1. Selected NOE (red) and HMBC (black) correlations for **1**.

Long-range correlations between NH/NCH$_3$ protons to carbonyl carbons of adjacent amino acids allowed us to establish the following sequences: AHMHA-Asn-*o*Tyr-γMePro and NMeGly-dAbu. Moreover, connectivity of the γMePro unit to ThrOMe residue was indicated from an HMBC correlation between one of the γMePro-δ-methylene protons (δ 4.23) and the carbonyl at δ 174.3 (C-1 ThrOMe).

Definitive confirmation of the sample structure was derived from ESI MS/MS analysis. In addition to the pseudomolecular ion at *m*/*z* 945.81 $[M+H]^+$, the ESI Q-TOF MS/MS spectrum provided several fragment ion peaks. The detailed interpretation of the fragmentation pattern confirmed both the sequence of amino acids and the identity of the amidic-bearing amino acid residues. The major peaks correspond to *C*-terminus fragments derived from a homogeneous ring opening between the dehydrated threonine (dAbu) and the *N*-methylglycine residue.

In particular, the signal at *m*/*z* 744.55 corresponding to the loss of 201.26 amu from the pseudomolecular ion was indicative of the presence of *N*-methylglycine and β-hydroxyaspartic acid residues. Subsequently, the loss of 114.99 amu from the pseudo y_6 ion was attributed to the lack of ThrOMe (*m*/*z* at 629.56) residue, and the further losses of 111.30 amu from pseudo y_5 and 163.07 amu from pseudo y_4 were indicative of the presence of γ-methylproline and *o*-tyrosine.

Besides, several internal fragments have been detected; the peak at 275.21 amu has been attributed to the dipeptide unit γ-methylproline/*o*-tyrosine; the fragment at 389.30 amu was ascribed to the tripeptide γMePro-*o*Tyr-Asn and the peak at 546.51 to the previous tripeptide increased by AHMHA unit. Finally, the peak at 629.88 amu supported the presence of the dAbu residue.

The absolute stereochemistry of Asn and ThrOMe residues was determined by acid hydrolysis of perthamide C (**1**) (6 N HCl, 110 °C, 12 h) and Marfey's analysis. The acid hydrolysate was derivatised with 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (L-FDAA), and LC–MS comparison of the derivatives from parent peptide with the FDAA derivatives of appropriate standards established the presence of L-Asn and L-ThrOMe.

The relative and absolute stereochemistry of the non conventional amino acid residues was determined on the basis of spectroscopic, chromatographic methods and by comparison with literature data.

ROESY correlations (in CD$_3$OH) between the –NH signal at δ 9.08 and the olefinic proton H-3 at δ 5.96 indicated the (*E*)-geometry for dAbu unit. Both geometrical isomers of 2-amino-2-butenoic unit have been found as components of marine peptides,[35–38] and have a distinct pattern of chemical shift values.[39] The ^{1}H- and ^{13}C NMR chemical shifts of C3 nuclei corroborate the proposed stereochemistry.

The relative configuration of γ-methylproline was determined on the isolated amino acid residue obtained by acidic hydrolysis of the parent peptide followed by HPLC separation.

Recently a stereoselective access to all stereoisomers of γ-methylproline residue has been published together with relevant physical data.[40] The two diastereoisomers can be differentiated on the basis of ^{1}H NMR data. Particular diagnostic are the chemical shifts of H-3 diasterotopic methylene protons observed at δ_H 2.52 and 1.64 in the *cis* isomer and at δ_H 1.94 and 2.25–2.38 in the *trans* isomer, and the coupling constant pattern of one of the H-5 protons observed as a broad triplet (δ_H 2.87, *J* 10.6) in the *cis* isomer and as double doublet (δ_H 2.83, *J* 11.5 and 8.6 Hz) in the *trans* isomer. The ^{1}H NMR spectral data of γ-methylproline isolated from parent perthamide C (see Experimental section) are consistent with the *cis* stereochemistry.

The absolute configuration of the γ-methylproline residue was determined by application of Marfey's method. Marfey's derivatives of all stereoisomers of γ-methylproline has been prepared and characterised, and it was found that they follow the usual elution order, namely the L-amino acid FDAA derivative is eluted from a C-18 column before its corresponding D-isomer.[40] Thus a small sample of perthamide C (**1**) was hydrolysed and derivatised with both enantiomers of Marfey's reagent. By monitoring for FDAA-γ-methylproline at *m*/*z* 382, the 2S configuration was assigned, therefore we assign the *cis*-4-methyl-L-proline stereostucture to this subunit.

J-Based NMR configurational analysis method was used to determine the relative configuration of β-hydroxyasparagine residue (Fig. 2). To support the assignment of an *anti* relative configuration and to assign the absolute stereochemistry, all stereoisomers of β-hydroxyaspartate were prepared by ozonolysis of the corresponding β-hydroxyphenylalanine stereoisomers.[41] The so obtained β-hydroxyaspartic acids were used as standards in the Marfey's analysis, that evidenced the presence of (2*R*,3*S*)-βOHAsn residue in

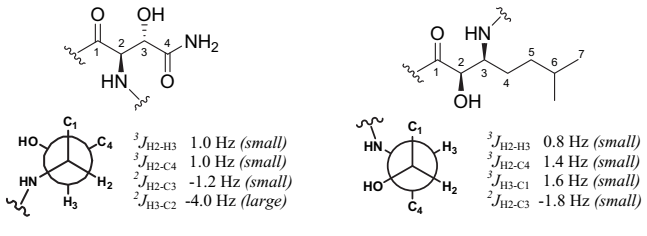


Figure 2. Relative configuration for AHMHA and βOHAsn residues in perthamide C (**1**).

perthamide C. Even if the heavy overlapping in the corresponding proton region made the measurement of coupling constants difficult, the relative disposition of the hydroxyl and the amino group on the α- and β-position, respectively, of the new 3-amino-2-hydroxy-6-methylheptanoic acid (AHMHA) was tentatively assigned on the basis of the *J*-based NMR configurational analysis method. A small coupling constant between H-2 and H-3 (0.8 Hz) suggested that they were *gauche*. H-2 exhibited a small ^3J coupling constant with C-4, and H-3 displayed a small ^3J coupling constant with C-1, indicating that H-2/C-4 and H-3/C-1 were *gauche*, pointing for a *threo* configuration. The stereoselective synthesis of AHMHA residue is currently in progress in our laboratory, thus the absolute stereochemistry of the above residue remained undetermined.

The absolute configuration of the new *o*Tyr residue was assigned after transformation of the residue, isolated from the acidic hydrolysis of perthamide C (see Experimental section), to aspartate by ozonolysis followed by oxidative work-up. The presence of a peak corresponding to L-Asp was observed by ion-selective monitoring for FDAA-Asp (*m*/*z* 386) indicating that *o*Tyr had a L-configuration.

The molecular formula of perthamide D (**2**), deduced by HR ESIMS, was 16 mass units (one oxygen atom) smaller than that of perthamide C (**1**). The ^{1}H NMR spectrum, which is very similar to that of **1** clearly evidenced the presence of a monosubstituted phenyl ring, suggesting the replacement of *o*-tyrosine residue in **2** with a phenylalanine residue. Interpretation of 2D NMR data, including COSY, HSQC, HMBC and ROESY data (Table 1) led to the planar structure of **2**. The complete agreement of chemical shifts and coupling constant patterns of AHMHA and dAbu residues suggested the same configuration. Application of Marfey's method enable us to determine the chirality of the residues as L-Asn, L-Phe, L-ThrOMe, *cis*-4-methyl-l-proline and (2*R*,3*S*)-βOHAsp.

Perthamides C and D did not show antiproliferative activity on KB cell line up to a dose of 10 μg/mL. However, when tested in a well characterised model of inflammation in vivo, i.e., mouse paw oedema,[42] perthamide C significantly reduced carrageenan-induced paw oedema both in the early phase (0–6 h) and in the late phase (24–96 h) as shown in Figure 3. In particular perthamide C displayed a dose-dependent (0.1, 0.3 and 1 mg/kg) anti-inflammatory activity causing about 60% reduction of oedema in mice at the dose of 300 μg/kg (ip). Also perthamide D showed an anti-inflammatory activity in both phases of oedema; however due to the scarcity of natural compound available, it was tested only in a single dose (0.3 mg/kg) where caused a 46% oedema inhibition. These data, if compared with the ED_{50} of one of the most common NSAID sold in the market, e.g., naproxen (ED_{50} 40 mg/kg), clearly indicate that perthamide C is about 100 times more potent. Furthermore the activity observed implies that the peptide is bioavailable and able to access the site of inflammation.

Anti-inflammatory marine cyclic peptides are rare, with the exception of cyclomarins,[43] salinamides[44] and halipeptins;[45] the discovery that perthamides are potent and, in a way, unexpected anti-inflammatory agents deserves further investigations on the origin of their activity.

Detailed pharmacological investigations to clarify the biomolecular target and therefore the mechanism of action at the molecular level are currently in progress in our laboratory. Indeed the modular peptidic nature and the ready chemical access to perthamide C may open the possibility to investigate new pharmacophores useful in the identification of new leads for developing a new generation of anti-inflammatory drugs.

3. Experimental section

3.1. General experimental procedures

Specific rotations were measured on a Perkin–Elmer 243 B polarimeter. High-resolution ESIMS spectra were performed with a Micromass QTOF Micro mass spectrometer. ESIMS experiments were performed on an Applied Biosystem API 2000 triple-quadrupole mass spectrometer. NMR spectra were obtained on Varian Inova 500 and Varian Inova 700 NMR spectrometers (^{1}H at 500 and 700 MHz, ^{13}C at 125, and 175 MHz, respectively) equipped with a Sun hardware, δ (ppm), *J* in hertz, spectra referred to CD_3OH (δ_H=3.31) as internal standard. HPLC was performed using a Waters Model 510 pump equipped with Waters Rheodine injector and a differential refractometer, model 401.

All reagents were commercially obtained (Aldrich, Fluka) at the highest commercial quality and used without further purification except where noted. All reactions were monitored by TLC on silica gel plates (Macherey–Nagel).

3.2. Sponge material and separation of individual peptides

T. swinhoei (order Lithistida, family Theonellidae) was collected on the barrier reef of Vangunu Island, Solomon Islands, in July 2004. The samples were frozen immediately after collection and lyophilised to yield 207 g of dry mass. Taxonomic identification was performed by Prof. John Hooper of Queensland Museum, Brisbane, Australia, and reference specimens are on file (R3170) at the ORSTOM Centre of Noumea.

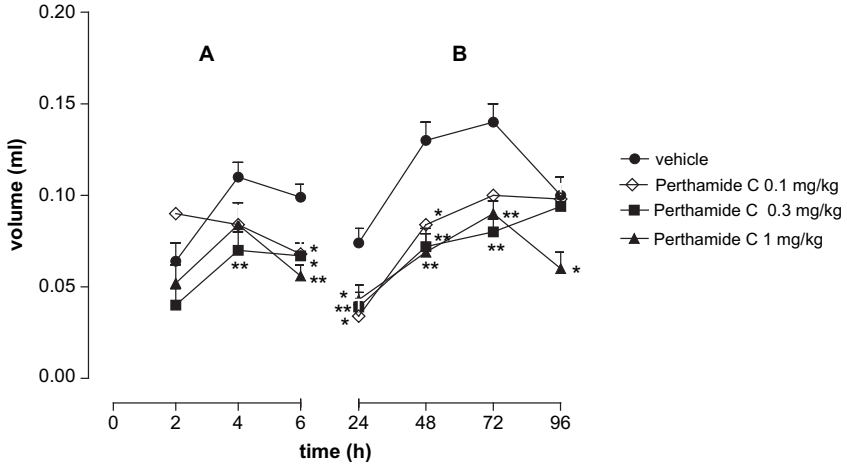


Figure 3. Dose-dependent inhibition on first (A) and second (B) phase of carrageenan-induced paw oedema by perthamide C (**1**).

The lyophilised material (207 g) was extracted with methanol (3×1.7 L) at room temperature and the crude methanolic extract was subjected to a modified Kupchan's partitioning procedure as follows. The methanol extract was dissolved in a mixture of MeOH/H_2O containing 10% H_2O and partitioned against *n*-hexane. The water content (% v/v) of the MeOH extract was adjusted to 30% and partitioned against $CHCl_3$. The aqueous phase was concentrated to remove MeOH and then extracted with *n*-BuOH.

The *n*-BuOH extract (4 g) was chromatographed in two runs by DCCC using *n*-BuOH/Me_2CO/H_2O (3:1:5) in the descending mode (the upper phase was the stationary phase), flow rate 8 mL/min; 4 mL fractions were collected and combined on the basis of their similar TLC retention factors.

Fractions 8–12 (560 mg) was purified by HPLC on a Phenomenex Hydro column (10 μ, 250×10 mm, 3.0 mL/min) with 24% CH_3CN/H_2O as eluent to give 276 mg of perthamide C (**1**) (t_R=11.7 min). Fraction 8 showed an addition peak at t_R=12.6 min in the HPLC trace containing a mixture of **1** and **2**. This latter was purified by HPLC on Vydac C18 column (5 μ, 250×4.6 mm, 1.0 mL/min) with 20% CH_3CN/H_2O as eluent to give 2.1 mg of perthamide D (**2**) (t_R=10.1 min).

3.3. Characteristic data for each compound

*3.3.1. Perthamide C (**1**).* White amorphous solid; $[\alpha]_D^{25}$ −6.3 (*c* 2.8, chloroform); ^1H and ^{13}C NMR data in CD_3OH given in Table 1; ESIMS: *m*/*z* (%) 945.8 $[M+H]^+$. HRMS (ESI): calcd for $C_{43}H_{65}N_{10}O_{14}$: 945.4682; found 945.4652 $[M+H]^+$.

*3.3.2. Perthamide D (**2**).* White amorphous solid; $[\alpha]_D^{25}$ −4.1 (*c* 0.1, chloroform); ^1H and ^{13}C NMR data in CD_3OH given in Table 1; ESIMS: *m*/*z* (%) 929.5 $[M+H]^+$. HRMS (ESI): calcd for $C_{43}H_{65}N_{10}O_{13}$: 929.4733; found 929.4773 $[M+H]^+$.

3.4. Amino acid analysis of perthamides

For a large scale hydrolysis, a 12 mg sample of perthamide C (**1**) dissolved in 6 N HCl (3 mL) and heated at 130 °C for 12 h. The crude residue was fractionated by HPLC on the reversed-phase Phenomenex Hydro (4 μ, 250×4.6 mm) column eluting with MeOH/H_2O 2:98, (flow rate 0.5 mL/min) to give: NMeGly (t_r=4.8 min), ThrOMe (t_r=6.4 min), γMePro (t_r=7.6 min), *o*Tyr (t_r=24.6 min).

γMePro: ^1H NMR (500 MHz, D_2O) δ 4.10 (1H, dd, *J*=6.7, 5.1 Hz, H-2), 3.44 (1H, dd, *J*=10.5, 7.6 Hz, H-5a), 2.90 (1H, t, *J*=10.5 Hz, H-5b), 2.51 (1H, dt, *J*=13.0, 7.0 Hz, H-3a), 1.60 (1H, dt, *J*=13.0, 9.4 Hz, H-3b), 1.04 (3H, d, *J*=6.6 Hz, Me-4).

3.5. Determination of the absolute configuration

3.5.1. Peptide hydrolysis. Peptide samples (200 μg) were dissolved in degassed 6 M HCl (0.5 mL) in an evacuated glass tube and heated at 160 °C for 16 h. The solvent was removed in vacuo and the resulting material was subjected to further derivatisation.

3.5.2. LC–MS analysis of Marfey's (FDAA) derivatives. A portion of the hydrolysate mixture (800 μg) or the amino acid standard (500 μg) was dissolved in 80 μL of a 2:3 solution of TEA:MeCN and this solution was then treated with 75 μL of 1% 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (FDAA) in 1:2 MeCN:acetone. The vials were heated at 70 °C for 1 h, and the contents were neutralised with 0.2 N HCl (50 μL) after cooling to room temperature. An aliquot of the L-FDAA derivative was dried under vacuum, diluted with MeCN-5% HCOOH in H_2O (1:1), and separated on a Proteo C18 (25×1.8 mm i.d.) column by means a linear gradient from 10%–50% aqueous acetonitrile containing 5% formic acid and 0.05% trifluoroacetic acid, over 45 min at 0.15 mL/min. The RP-HPLC system was connected to the electrospray ion source by inserting a splitter valve and the flow going into the mass spectrometer source was set at a value of 100 μL/min. Mass spectra were acquired in positive ion detection mode (*m*/*z* interval of 320–900) and the data were analysed using the suite of programs Xcalibur; all masses were reported as average values. Capillary temperature was set at 280 °C, capillary voltage at 37 V, tube lens offset at 50 V and ion spray voltage at 5 V.

Retention times (min) of FDAA-amino acids are given in parentheses: D-Asp (26.5), L-Asp (25.2), D-*allo*ThrOMe (37.3), L-*allo*ThrOMe (32.5), D-ThrOMe (39.8), L-ThrOMe (34.5) (2*S*,3*R*)-βOHAsp (24.0), (2*R*,3*S*)-βOHAsp (21.8), (2*S*,3*S*)-βOHAsp (18.0), (2*R*,3*R*)-βOHAsp (16.9), L-Phe (45.0), D-Phe (49.2).

The hydrolysate of perthamide C (**1**) contained: L-Asp (25.4), L-ThrOMe (34.5), (2*R*,3*S*)-βOHAsp (21.9).

The hydrolysate of perthamide D (**2**) contained: L-Asp (25.4), L-ThrOMe (34.5), (2*R*,3*S*)-βOHAsp (21.9), L-Phe (45.0).

To determine the absolute configuration of γMePro in perthamides C and D, two aliquots of the hydrolysate mixtures were derivatised with L- and D-FDAA, respectively and then they were subjected to LC–MS as described above. Retention times (min): L-FDAA- γMePro (37.7), D-FDAA- γMePro (39.8).

To determine the absolute configuration of *o*Tyr in perthamide C, a stream of ozone in O_2 was bubbled through cooled solutions of pure amino acid residue (0.1 mg) in MeOH (0.5 mL) at −78 °C for 1 h. Hydrogen peroxide (35%, 10 drops) was added to the reaction mixture, which was then allowed to stand at room temperature overnight. The solvent was removed under a stream of N_2 and the ozonolysis product was subjected to Marfey's derivatisation with L-FDAA and LC–MS analysis to obtain L-Asp (25.4 min).

3.6. Pharmacological assays

3.6.1. Mouse paw oedema. Male Swiss (CD-1; Harlan, Italy) weighing 28–30 g were divided into groups (*n*=6 each group) and lightly anaesthetized with isoflurane. Each group of animals received subplantar injection of 50 μL of carrageenan 1% (w/v) or 50 μL of saline in the left hind paw. Paw volume was measured by using an hydropletismometer specially modified for small volumes (Ugo Basile, Comerio, Italy) immediately before the subplantar injection and 2, 4, 6, 24, 48, 72 and 96 h thereafter. The same operator always performed the double-blind assessment of paw volume. The increase in paw volume was calculated as the difference between the paw volume measured at each time point and the basal paw oedema. Each group of animals received intraperitoneal administration of Perthamide C (0.1, 0.3, 1 mg/kg), Perthamide D (0.3 mg/kg) or vehicle (PEG). All drugs were administrated immediately before the injection of carrageenan and 24 h thereafter.

3.6.2. Statistical analysis. Results were expressed as mean±s.e.m. Statistical analysis was determined by one way ANOVA followed by Dunnett's test for multiple comparisons, using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Differences were considered statistically significant when $p<0.05$.

Acknowledgements

This work was supported by grants from MIUR (PRIN 2007) 'Sostanze Naturali ed Analoghi Sintetici con Attività Antitumorale' Rome, Italy. NMR spectra were provided by the CSIAS, Centro Interdipartimentale di Analisi Strumentale, Faculty of Pharmacy, University of Naples. We thank Dr. John Hooper for the identification of the sponge. This work is part of the C2 component of the CRISP (Coral Reef Initiative in the South Pacific) project and granted by the Agence Française de Développement. We thank the Solomon

Islands government for permitting us to collect in their country, the Fisheries Department and R. Sulu (University of the South Pacific in Honiara) for their help and assistance. We acknowledge Prof. C. Payri (IRD - Université de Polynésie Française) for her essential contribution to the field trip.

References and notes

1. Hamada, T.; Matsunaga, S.; Yano, G.; Fusetani, N. *J. Am. Chem. Soc.* **2005**, *127*, 110–118.
2. Fusetani, N.; Warabi, K.; Nogata, Y.; Nakao, Y.; Matsunaga, S.; Van Soest, R. R. M. *Tetrahedron Lett.* **1999**, *40*, 4687–4690.
3. Araki, T.; Matsunaga, S.; Fusetani, N. *Biosci. Biotechnol. Biochem.* **2005**, *69*, 1318–1322.
4. Fusetani, N.; Matsunaga, S.; Matsumoto, H.; Takebayashi, Y. *J. Am. Chem. Soc.* **1990**, *112*, 7053–7054.
5. Nakao, Y.; Matsunaga, S.; Fusetani, N. *Bioorg. Med. Chem.* **1995**, *3*, 1115–1122.
6. Nakao, Y.; Oku, N.; Matsunaga, S.; Fusetani, N. *J. Nat. Prod.* **1998**, *61*, 667–670.
7. Kobayashi, J.; Sato, M.; Murayama, T.; Ishibashi, M.; Walchi, M. R.; Kanai, M.; Shoji, J.; Ohizumi, Y. *J. Chem. Soc., Chem. Commun.* **1991**, 1050–1052.
8. Fusetani, N.; Sugawara, T.; Matsunaga, S.; Hirota, H. *J. Am. Chem. Soc.* **1991**, *113*, 7811–7812.
9. Roy, M. C.; Ohtani, I. I.; Tanaka, J.; Higa, T.; Satari, R. *Tetrahedron Lett.* **1999**, *40*, 5373–5376.
10. Kobayashi, J.; Sato, M.; Ishibashi, M.; Shigemori, H.; Nakamura, T.; Ohizumi, Y. *J. Chem. Soc., Perkin Trans. 1* **1991**, 2609–2611.
11. Itagaki, F.; Shigemori, H.; Ishibashi, M.; Nakamura, T.; Sasaki, T.; Kobayashi, J. *J. Org. Chem.* **1992**, *57*, 5540–5542.
12. Kobayashi, J.; Itagaki, F.; Shigemori, H.; Takao, T.; Shimonishi, Y. *Tetrahedron* **1995**, *51*, 2525–2532.
13. Uemoto, H.; Yahiro, Y.; Shigemori, H.; Tsuda, M.; Takao, T.; Shimonishi, Y.; Kobayashi, J. *Tetrahedron* **1998**, *54*, 6719–6724.
14. Tsuda, M.; Ishiyama, H.; Masuko, K.; Takao, T.; Shimonishi, Y.; Kobayashi, J. *Tetrahedron* **1999**, *55*, 12543–12548.
15. Bonnington, L. S.; Tanaka, J.; Higa, T.; Kimura, J.; Yoshimura, Y.; Nakao, Y.; Yoshida, W. Y.; Scheuer, P. J. *J. Org. Chem.* **1997**, *62*, 7765–7767.
16. Chill, L.; Kashman, Y.; Schleyer, M. *Tetrahedron* **1997**, *53*, 16147–16152.
17. Matsunaga, S.; Fusetani, N.; Hashimoto, K.; Walchli, M. *J. Am. Chem. Soc.* **1989**, *111*, 2582–2588.
18. Kobayashi, M.; Kanzaki, K.; Katayama, S.; Ohashi, K.; Okada, H.; Ikegami, S.; Kitagawa, I. *Chem. Pharm. Bull.* **1994**, *42*, 1410–1415.
19. Kitagawa, I.; Kobayashi, M.; Lee, N. K.; Shibuya, H.; Kawata, Y.; Sakiyama, F. *Chem. Pharm. Bull.* **1986**, *34*, 2664–2667.
20. Clark, D. P.; Carroll, J.; Naylor, S.; Crews, P. J. *Org. Chem.* **1998**, *63*, 8757–8764.
21. Araki, T.; Matsunaga, S.; Nakao, Y.; Furihata, K.; West, L.; Faulkner, D. J.; Fusetani, N. *J. Org. Chem.* **2008**, *73*, 7889–7894.
22. Ford, P. W.; Gustafson, K. R.; McKee, T. C.; Shigematsu, N.; Maurizi, L. K.; Pannell, L. K.; Williams, D. E.; Dilip de Silva, E.; Lassota, P.; Allen, T. M.; Van Soest, Rob; Andersen, R. J.; Boyd, M. R. *J. Am. Chem. Soc.* **1999**, *121*, 5899–5909.
23. Okada, Y.; Matsunaga, S.; van Soest, R. W. M.; Fusetani, N. *Org. Lett.* **2002**, *4*, 3039–3042.
24. Ratnayake, A. S.; Bugni, T. S.; Feng, X.; Harper, M. K.; Skalicky, J. J.; Mohammed, K. A.; Andjelic, C. D.; Barrows, L. R.; Ireland, C. M. *J. Nat. Prod.* **2006**, *69*, 1582–1586.
25. Bewley, C. A.; Faulkner, D. J. *J. Org. Chem.* **1994**, *59*, 4849–4852.
26. Schmidt, E. W.; Bewley, C. A.; Faulkner, D. J. *J. Org. Chem.* **1998**, *63*, 1254–1258.
27. Bewley, C. A.; Holland, N. D.; Faulkner, D. J. *Experientia* **1996**, *52*, 716–722.
28. Schmidt, E. W.; Obraztsova, A. Y.; Davidson, S. K.; Faulkner, D. J.; Haygood, M. G. *Marine Biol.* **2000**, *136*, 969–977.
29. Bewley, C. A.; Faulkner, D. J. *Angew. Chem., Int. Ed.* **1998**, *37*, 2162–2178.
30. Matsunaga, S.; Fusetani, N. *Curr. Org. Chem.* **2003**, *7*, 945–966.
31. Gulavita, N. K.; Pomponi, S. A.; Wright, A. E.; Yarwood, D.; Sills, M. A. *Tetrahedron Lett.* **1994**, *35*, 6815–6818.
32. Kupchan, S. M.; Britton, R. W.; Ziegler, M. F.; Sigel, C. W. *J. Org. Chem.* **1973**, *38*, 178–179.
33. Karam, L. R.; Simic, M. G. *J. Biol. Chem.* **1990**, *265*, 11581–11585.
34. Ming, D.; Wie, W.; Nuran, E.; Yinfa, M. *J. Chromatogr., B* **2004**, *803*, 321–329.
35. Sano, T.; Kaya, K. *Tetrahedron Lett.* **1995**, *36*, 8603–8606.
36. Sano, T.; Kaya, K. *Tetrahedron* **1998**, *54*, 463–470.
37. Sano, T.; Beattie, K. A.; Codd, G. A.; Kaya, K. *J. Nat. Prod.* **1998**, *61*, 851–853.
38. Bewley, C. A.; He, H.; Williams, D. H.; Faulkner, D. J. *J. Am. Chem. Soc.* **1996**, *118*, 4314–4321.
39. Sepe, V.; D'Orsi, R.; Borbone, N.; D'Auria, M. V.; Bifulco, G.; Monti, M. C.; Catania, A.; Zampella, A. *Tetrahedron* **2006**, *62*, 833–840.
40. Murphy, A. C.; Mitova, M. I.; Blunt, J. W.; Munro, M. H. G. *J. Nat. Prod.* **2008**, *71*, 806–809.
41. Easton, C. J.; Hutton, C. A.; Roselt, P. D.; Tiekink, E. R. T. *Tetrahedron* **1994**, *50*, 7327–7330.
42. Posadas, I.; Bucci, M.; Roviezzo, F.; Rossi, A.; Parente, L.; Sautebin, L.; Cirino, G. *Br. J. Pharmacol.* **2004**, *142*, 331–338.
43. Renner, M. K.; Shen, Y. C.; Cheng, X. C.; Jensen, P. R.; Frankmoelle, W.; Kauffman, C. A.; Fenical, W.; Lobkovsky, E.; Clardy, J. *J. Am. Chem. Soc.* **1999**, *121*, 11273–11276.
44. (a) Trischman, J. A.; Tapiolas, D. M.; Jensen, P. R.; Dwight, R.; Fenical, W.; McKee, T. C.; Ireland, C. M.; Stout, T. J.; Clardy, J. *J. Am. Chem. Soc.* **1994**, *116*, 757–758; (b) Moore, B. S.; Trischman, J. A.; Seng, D.; Kho, D.; Jensen, P. R.; Fenical, W. *J. Org. Chem.* **1999**, *64*, 1145–1150.
45. Randazzo, A.; Bifulco, G.; Giannini, C.; Bucci, M.; Debitus, C.; Cirino, G.; Gomez-Paloma, L. *J. Am. Chem. Soc.* **2001**, *123*, 10870–10876.