A Novel Cyclopeptide from a Bacterium Associated with the Marine Sponge *Ircinia muscarum*

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A new cyclotetrapeptide **1**, together with three known cyclopeptides were isolated from the exo-cellular extract of *Pseudomonas* sp. a bacterium associated with the sponge *Ircinia muscarum.* The structure of **1** was suggested on the basis of spectroscopic analytical data and chemical degradation.

Key words: Marine Bacteria, Cyclopeptide, Sponge

Introduction

Marine microorganisms are of considerable current interest as a new and promising source of biologically active compounds many of which can be used for drug development (Fenical, 1993; Kobayashi and Ishibashi, 1993). Special interest is pointed to the peptides and modified peptides with origin from marine microorganisms (Faulkner, 2002). An intriguing group of bioactive peptides are cyclic peptides, which exhibit a wide range of biological activities including cytotoxic, antibiotic and antifungal activity (Ireland *et al.*, 1989; Fusetani and Matsunaga, 1993), activity on central nervous system (Prasad, 1995).

Marine sponges are a rich source of novel microorganisms with potential pharmacological activity (Hentschel *et al.*, 2001). The surfaces and internal spaces of sponges provide a specialised environmental niche that contains high numbers of bacteria exceeding those of seawater by two or three orders of magnitude (Friedrich *et al.*, 2001). Moreover interest to sponge-associated bacteria recently has increased since several studies prove that the real producer of bioactive compounds initially isolated from marine sponges are associated commensally or symbiotically microorganisms (Proksch *et al.*, 2002).

Sponge peptides are also assumed to be of microbial origin due to the presence of both D-amino acids and unusual amino acids (Fusetani and Matsunaga, 1993). Diketopiperazines ascribed to the sponge *Tedania ignis* (Schmitz *et al.*, 1983) were shown to be produced by the associated bacterium *Micrococcus* sp. (Stierle *et al.*, 1988), and theopalauamide, a cyclic glycopeptide isolated from the sponge *Theonella swinhoei* was proven to be included in uncultivable symbiont "*Candidatus* Entotheonella palauensis" (Schmidt *et al.*, 2000).

Continuing our search for new bioactive compounds from microorganisms associated with marine sponges, we have previously reported a novel β-amino acid containing tripeptide isolated from a *Pseudomonas-Alteromonas* bacterium associated with the marine sponge *Dysidea fragilis* (De Rosa *et al.*, 2000) and a novel cyclic tetrapeptide isolated from a *Pseudomonas* sp. (strain IM-1) associated with the marine sponge *Ircinia muscarum* (De Rosa *et al.*, 2002). In this study we report the elucidation of exo-cellular peptide composition of strain IM-1.

Materials and Methods

General experimental procedures

Optical rotation was measured on a JASCO DIP 370 polarimeter, using a 10-cm microcell. FABMS were obtained on a VG-ZAB instrument equipped with a FAB source at 25 KeV (2 μA), using glycerol as a matrix. EIMS were recorded on a JEOL JMS D-300. ^{1}H and ^{13}C-NMR spectra were recorded at 500 and 125 MHz, respectively, on a Bruker AMX-500 spectrometer in D_2O and/or CD_3OD, using the residual D_2O or CD_3OD resonance at 4.78 ppm, 3.48 ppm and 49.0 ppm as internal references, respectively.

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Bacterial isolation

I. muscarum (Dictyoceratida, Spongiidae) collected in the bay of Naples (Italy) at a depth of 20 m, was covered by seawater and transported immediately to the laboratory. Water samples were collected at the same time and place. The sponge was rinsed in sterile natural seawater (0.22 μm filtered), and then transferred into new portion of sterile seawater and the surface tissue was carefully removed from the inner tissue (mesohyl) with a scalpel blade. The mesohyl tissue was rinsed two more times in sterile seawater, cut into small pieces and ground in a porcelain mortar. The homogenates obtained were diluted in Marine broth (2216 DIFCO). Different dilutions were used: 10^{-1} (0.1 ml water or homogenate + 0.9 ml broth) 10^{-2}, 10^{-3} and 10^{-4}. A 0.1 ml aliquot of each dilution was plated on Marine agar (2216 DIFCO, pH 7.6). After a 72 h incubation at 19 °C single colonies of bacterial isolates were transferred to fresh medium and streaked repeatedly to fresh medium after 72 h of incubation to insure the purity of the culture. IM-1 (a voucher specimen of the strain is maintained in the ICB-CNR collection) was identified as *Pseudomonas* sp. The characterisation was done by routine biochemical tests (API 20E, identification system for Gram-negative bacteria, BioMérieux, Lyon, France), modified for marine bacteria.

Isolation and identification of cyclopeptides

Cultures (4 l) were incubated at 37 °C (optimal growth temperature) for 72 h under shaking. The cells were removed by centrifugation at 15,000 × *g* for 15 min. Then the culture medium was extracted first with ethylacetate (300 ml × 3) and after that with *n*-butanol (300 ml × 3) and the solvents evaporated under reduced pressure. The *n*-butanol extract was chromatographed on a Lobar RP-18 column (LiChroprep RP-18, 40–63 μm, 310 × 25 mm, Merck) with a water/methanol gradient (flow rate: 3 ml/min). Fractions of 9 ml were collected. Fractions 35 (5.5 mg) and 43–44 (8.6 mg) contained compounds **3** and **4**, respectively. Fractions 36–42 (33.4 mg) were additionally purified by reversed phase HPLC (Kromasil KR100–5-C18, 10 × 250 mm, Eka Chemicals, Bohus, Sweden; water/methanol gradient containing 0.1% trifluoracetic acid; flow: 2.5 ml/min; detection UV: 215 nm) to give compounds **1** (3.2 mg) and **2** (10.4 mg).

Cyclo-(L-prolyl-D-prolyl-L-tyrosyl-L-tyrosine) (**1**): white amorphous solid; $[\alpha]_D$ – 7.6° (*c* = 0.003, MeOH); NMR data are reported in Table I; HRFABMS *m/z* (%) 521.2391 (38) $(M + H)^+$ (calculated for $C_{28}H_{33}N_4O_6$, 521.2400), 261 (100).

Cyclo-(L-leucyl-*cis*-4-hydroxy-D-prolyl-L-leucyl-*trans*-4-hydroxy-L-proline) (**2**): white amorphous solid; $[\alpha]_D$ – 18.1° (*c* = 0.011, MeOH); ^{1}H NMR (CD_3OD) δ 4.56 (1H, dd, *J* = 11.2 and 6.5 Hz, H-α of 4Hyp-1), 4.50 (1H, m, H-γ of 4Hyp-1), 4.46 (1H, m, H-γ of 4Hyp-2), 4.39 (1H, dd, *J* = 8.1 and 7.9 Hz, H-α of 4Hyp-2), 4.21 (1H, dd, *J* = 5.5 and 5.3 Hz, H-α of Leu-2), 3.91 (1H, dd, *J* = 9.6 and 5.6 Hz, H-α of Leu-1), 3.70 (2H, ddd, *J* = 12.7, 4.3 and 3.0, H-δ of 4Hyp-2), 3.49 (2H, ddd, *J* = 12.2, 7.0 and 5.2, H-δ of 4Hyp-1), 2.52 (1H, ddd, *J* = 13.7, 8.1 and 5.5, H-β of 4Hyp-2), 2.32 (1H, ddd, *J* = 13.4, 6.5 and 2.5, H-β of 4Hyp-1), 2.27 (1H, ddd, *J* = 13.7, 7.9 and 2.4, H-β of 4Hyp-2), 2.13 (1H, ddd, *J* = 13.4, 11.2 and 4.3, H-β of 4Hyp-1), 1.94 (2H, m, H-β and H-γ of Leu-2), 1.82 (1H, m, H-γ of Leu-1), 1.72 (1H, ddd, *J* = 14.6, 9.6 and 5.5, H-β of Leu-1), 1.63 (1H, ddd, *J* = 14.6, 8.3 and 5.6, H-β of Leu-1), 1.03 (3H, d, *J* = 6.6, H-δ of Leu-1), 1.00 (6H, d, *J* = 6.4, H-δ of Leu-2), 0.99 (3H, d, *J* = 6.6, H-δ of Leu-1); HRFABMS *m/z* (%) 453.2714 (48) $(M + H)^+$ (calculated for $C_{22}H_{37}N_4O_6$, 453.2704), 341 (70), 227 (100).

Cyclo-(L-leucyl-*cis*-4-hydroxy-D-proline) (**3**): $[\alpha]_D$ – 49.6° (*c* = 0.003, MeOH); ^{1}H-NMR (D_2O): δ 4.56 (1H, m, H-8), 4.46 (1H, dd, *J* = 8.3 and 8.1 Hz, H-6), 4.00 (1H, m, H-3), 3.65 (1H, dd, *J* = 12.3 and 3.5 Hz, H-9a), 3.49 (1H, dd, *J* = 12.3 and 5.6 Hz, H-9b), 2.53 (1H, m, H-7a), 2.17 (1H, m, H-7b), 1.70 (2H, m, H-10), 1.54 (1H, m, H-11), 0.94 (3H, d, *J* = 6.1 Hz, H-12), 0.91 (3H, d, *J* = 6.1 Hz, H-13); EIMS *m/z* (%) $[M]^+$ 226 (6), 183 (12), 170 (100).

Cyclo-(L-phenylalanyl-*cis*-4-hydroxy-D-proline) (**4**): $[\alpha]_D$ + 10.4° (*c* = 0.002, MeOH); ^{1}H-NMR (D_2O): δ 7.34 (3H, m, H-13–15), 7.21 (2H, m, H-12 and H-16), 4.41 (1H, t, *J* = 4.4 Hz, H-3), 4.33 (1H, m, H-8), 3.58 (1H, dd, *J* = 12.4 and 3.8 Hz, H-9a), 3.34 (1H, dd, *J* = 12.4 and 5.7 Hz, H-9b), 3.22 (1H, dd, *J* = 13.6 and 4.2 Hz, H-10a), 3.06 (1H, dd, *J* = 13.6 and 5.0 Hz, H-10b), 2.62 (1H, dd, *J* = 8.5 and 8.4 Hz, H-6), 2.26 (1H, m, H-7a), 1.86 (1H, m, H-7b); EIMS *m/z* (%) $[M]^+$ 260 (15), 169 (86), 154 (33), 141 (100).

Amino acid analysis

Compounds **1–4** (200 μg) were hydrolysed with 6 N HCl (500 μl) at 110 °C for 1.3 h. The acid hydrolysate, divided into two parts, was dried under N_2. After derivatization with phenylisothiocyanate (PITC), a part, was used for amino acid analysis by PICO-TAG method (Waters, Milan, Italy). By co-injection with standard PTC amino acid derivatives the following amino acids were identified: Pro and Tyr for compound **1**, Leu and 4Hyp for compounds **2** and **3**, Phe and 4Hyp for compound **4**. The second part was subjected to chiral HPLC (Chirex D-Penicillamine; 250 × 4.6 mm, Phenomenex, Aschaffenburg, Germany; 30% MeOH in 2 mM $CuSO_4$, 1 ml/min) detected at 254 nm. By co-injection with standard amino acids the following amino acids were identified: L-Pro, D-Pro and L-Tyr for compound **1**, L-Leu, *trans*-L-4Hyp and *cis*-D-4Hyp for compound **2**, L-Leu and *cis*-D-4Hyp for compound **3**, L-Phe and *cis*-D-4Hyp for compound **4**.

Antimicrobial and antifungal assay

Gram-positive bacteria (*Bacillus subtilis* DSM 347, *Micrococcus luteus* DSM 498), Gram-negative bacterium (*Escherichia coli* DSM 498) and yeast (*Saccharomyces cerevisiae* DSM 70449) were used for the antimicrobial assays. The compounds **1–4** (150 μg each, dissolved in 0.1 ml of DMSO, final concentration 50 μg/ml; **1**: 96 μM, **2**: 111 μM, **3**: 221 μM, **4**: 192 μM) were individually added to appropriate medium (2.8 ml), in triplicate, and inoculated with (0.1 ml) stationary phase cultures of test microorganisms. Gentamycin (0.8 μg/ml) and nystatin (25 μg/ml) were used as controls. The bacterial and yeast growth was observed after a 48-h incubation at optimal growth temperature for each strain.

Cytotoxicity assays

The compounds **1–4** were tested against L 5178Y mouse lymphoma (DSMZ ACC 320), HeLa S3 cervical cancer (DSMZ ACC 161) and PC 12 pheochromocytoma (DSMZ ACC 159) cell lines at 5 μg/ml (**1**: 9.6 μM, **2**: 11 μM, **3**: 22 μM, **4**: 19 μM) as earlier reported (Müller *et al.*, 1987).

Results and Discussion

A specimen of *I. muscarum*, collected at 20 m depth in the gulf of Naples, was used for the isolation of bacteria. A strain of bacterium *Pseudomonas* sp. (IM-1) was isolated only from the sponge, but not from the surrounding water. The cultivation of the strain IM-1 on standard medium without any sponge-derived additives is an indication that this bacterium is not a symbiont, but is only specifically associated with the tissues of *I. muscarum.* The bacterium was grown in Marine broth at 37 °C, pH 7.6 for 72 h.

The *n*-butanol extract of the culture medium (4 l), after removal of bacteria, was purified repeatedly by Lobar RP-18, followed by reversed-phase HPLC to give four pure compounds. The structures of compounds **1–4** were elucidated by means of spectroscopic data 1D- and 2D-NMR, FABMS, EIMS and chiral HPLC analysis. Compound **2** cyclo-(L-leucyl–*cis*-4-hydroxy-D-prolyl-L-leucyl-*trans*-4-hydroxy-L-proline) was previously isolated from the same strain (De Rosa *et al.*, 2002). Two other compounds were identified as the known diketopiperazines cyclo-(L-leucyl-*cis*-4-hydroxy-D-proline) (**3**) and cyclo-(L-phenylalanyl-*cis*-4-hydroxy-D-proline) (**4**). Diketopiperazines **3** and **4** were previously isolated from a yeast associated with a marine sponge (Shigemori *et al.*, 1998). Compound **1** appeared to be a new cyclotetrapeptide.

Compound **1** had $[\alpha]_D$ − 7.6° (c = 0.003, MeOH) and showed a pseudomolecular ion peak at m/z 521.2391 (M + H^+, calculated 521.2400) in the

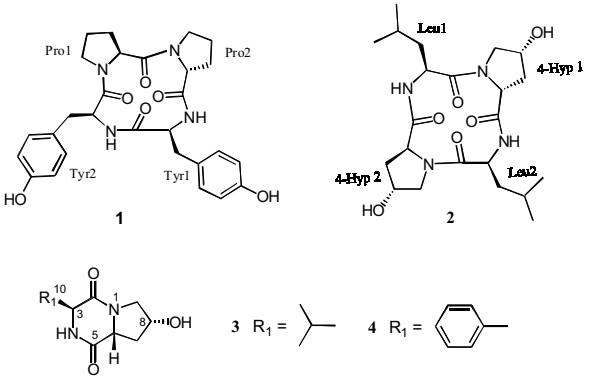

Fig. 1. Chemical structures of compounds **1–4**. Legend: Pro = proline; Tyr = tyrosine; Leu = leucine; 4-Hyp = 4-hydroxy-proline.

HRFABMS (positive ion) spectrum, consistent with a molecular formula $C_{28}H_{32}N_4O_6$. The peptide nature of this compound was suggested by the molecular formula itself and from analysis of its ^{1}H- and ^{13}C-NMR spectra. Detailed analyses of the ^{1}H- and ^{13}C-NMR spectral data (Table I) for **1**, with the aid of COSY-45, TOCSY, HMQC and HMBC spectra, established the presence of two tyrosine and two proline residues. The amino acid composition was confirmed by HPLC (PICO-TAG) analysis of the acid hydrolysate of **1** after derivatization with phenylisothiocyanate (PITC) which revealed the presence of tyrosine and proline. For this amino acid composition the molecular formula was in accordance with 15 degrees of unsaturation, requiring that **1** is a cyclic tetrapeptide.

The amino acid sequence of compound **1** was deduced by an interpretation of the HMBC spectrum and FABMS. The detailed interpretation of HMBC data allowed to assign the carbonyl signals of the four amino acid units. The signals at lowest field (δ 172.5 and 171.0) were assigned to the carbonyls of the proline units, the remaining signals (δ 167.8 and 166.9) to the two tyrosine units. HMBC correlations observed between the α proton of proline-1 (δ 3.99) and the carbonyl at δ 166.9 (tyrosine-2), α-proton of tyrosine 1 (δ 4.29) and the carbonyl of the second proline unit (δ 172.5) defined two amino acid sequences proline-tyrosine. The presence of a fragment at m/z 261 ($C_{14}H_{17}N_2O_3$) in FABMS confirms the presence of this unit in compound **1**. Further correlations of carbonyls of proline-1 (δ 171.0) and tyrosine-1

Table I. NMR spectra data of compound **1** in D_2O + CD_3OH solution[a].

	^{13}C	^{1}H	HMBC($J_{C\text{-}H}$ = 10 Hz)
Pro1			
α-CH	59.5	3.99 dd (6.0, 5.7)	H-β
β-CH_2	28.6	1.94 m	H-α, H-γ, H-δ
		0.68 ddd (11.6, 11.1, 9.2)	
γ-CH_2	21.7	1.72 m	H-β, H-δ
		1.61 m	
δ-CH_2	45.7	3.46 m	H-β, H-γ
		3.24 m	
CO	171.0		H-α, H-β
Pro2			
α-CH	58.9	2.39 dd (6.4, 4.0)	H-β, H-δ
β-CH_2	29.1	1.98 m	H-α, H-γ
		1.60 m	
γ-CH_2	21.9	1.87 m	H-β, H-δ
		1.58 m	
δ-CH_2	46.1	3.43 m	H-β, H-γ
		3.28 m	
CO	172.5	– –	H-α, H-β, H-α (Tyr1)
Tyr1			
α-CH	58.9	4.29 bt (4.0)	H-β
β-CH_2	39.4	2.91 dd (14.1, 4.9)	H-α, H-2, H-6
		3.11 dd (14.1, 3.7)	
1-C	127.3	– –	H-α, H-β, H-3, H-5
2,6-CH	132.4	7.00 δ (8.6)	Hβ
3,5-CH	116.5	6.80 δ (8.6)	H-2, H-6
4-C	155.7[b]	– –	H-2, H-3, H-5, H-6
CO	167.8	– –	Hα, Hβ
Tyr2			
α-CH	57.5	4.46 bs	H-β
β-CH_2	38.3	2.94 dd (14.4, 4.4)	H-2, H-6
		3.16 dd (14.4, 4.0)	
1-C	127.6	– –	
2,6-CH	132.4	7.00 δ (8.6)	H-β
3,5-CH	116.5	6.80 δ (8.6)	H-2, H-6
4-C	156.2[b]	– –	H-2, H-3, H-5, H-6
CO	166.9	– –	H-α, H-β, H-α (Pro1)

[a] Chemical shifts are referred to residual CD_3OD resonance. Multiplicities are indicated by usual symbols. Coupling constants (Hz) are in parentheses.

[b] Signals may be interchangeable.

(δ 167.8) were not observed. Considering the molecular formula $C_{28}H_{32}N_4O_6$ the structure of compound **1** requires the presence of a peptide bond between two proline and two tyrosine units, respectively.

The stereochemistry of the amino acids was determined by chiral HPLC analysis of the acid hydrolysate, which gave L-tyrosine and both L-proline and D-proline. The L- and D-stereochemistry of proline-1 and 2, respectively was assigned on the basis of chemical shifts of their α and β protons in comparison with those observed of two exo-cellular diketopiperazines [cyclo-(L-tyrosyl-L-proline) and cyclo-(L-tyrosyl-D-proline)] isolated from *Bacillus* sp. (IV-2) associated with the sponge *Ircinia variabilis* (De Rosa *et al.*, 2003). Thus the structure of compound **1** was elucidated as cyclo-(L-prolyl-D-prolyl-L-tyrosyl-L-tyrosine).

It is well-known that cyclic peptides can either be bio-synthesised by ribosomal or nonribosomal mechanisms. The nucleic acid-dependent ribosomal synthesis of peptides is restricted to the incorporation of only 21 proteinogenic amino acids. Nonribosomal mechanism of peptide synthesis is only limited by the length of the peptide chain formed, which is assembled from an exceedingly diverse group of precursors including pseudo, non-proteinogenic, hydroxy, N-methylated and D-amino acids (Marahiel *et al.*, 1997). Since cyclopeptides **1–4** contain D-amino acids is reasonable to assume a nonribosomal origin of these compounds.

In our assay the cyclopeptides **1–4** exhibited no cytotoxic, antimicrobial or antifungal activity.

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