This is a pre- or post-print of an article published in
Dipeptide cis-cyclo(Leucyl-Tyrosyl) produced by sponge
associated Penicillium sp. F37 inhibits biofilm formation
of the pathogenic Staphylococcus epidermidis
(2013) Bioorganic and Medicinal Chemistry Letters, 23
(3), pp. 624-626.
Dipeptide cis-cyclo(leucyl-tyrosyl) produced by sponge associated Penicillium sp.
F37 inhibits biofilm formation of the pathogenic Staphylococcus epidermidis

Marina Scopel¹, Wolf-Rainer Abraham², Amélia T. Henriques¹ and Alexandre J. Macedo¹,³

¹Faculdade de Farmácia, Departamento de Produção de Matéria-Prima, Universidade Federal do Rio Grande do Sul, Av. Ipiranga, 2752, 90610-000, Porto Alegre, Brazil.
²Helmholtz Centre for Infection Research, Chemical Microbiology, Braunschweig, Germany.
³Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves, 43431, 91501-970, Porto Alegre, Brazil.

*Corresponding author; Tel: +55 51 33085354, Fax: +55-51 33087309 alexandre.macedo@ufrgs.br
Abstract

Infections associated to microbial biofilms are involved in 80% of human infections and became a challenge concerning public health. Infections related to *Staphylococcus epidermidis* biofilms are presently commonly associated to medical devices, increasing treatment costs for this type of infection. Alternatives to eliminate this kind of disease have been employed in screening programs using diverse marine-derived fungi source of bioactive compounds capable to combat biofilm formation. In this work was isolated the dipeptide *cis*-cyclo(leucyl-tyrosyl) from a sponge associated *Penicillium sp.* possessing a remarkable inhibition up to 85% of biofilm formation without interfering with bacterial growth, confirmed by scanning electron microscopy. This is the first demonstration that *cis*-cyclo(leucyl-tyrosyl) is able to specifically inhibit biofilm formation adding another aspect to the broad spectrum of bioactivities of cyclic dipeptides.

**Key words** *cis*-cyclo(leucyl-tyrosyl), *Staphylococcus epidermidis*, biofilm, marine-derived fungi
Biofilm formation on medical devices, such as catheters, prosthesis, implants, and diseases, such as cystic fibrosis, osteomyelitis, endocarditis, otitis, prostatitis, periodontitis, conjunctivitis\textsuperscript{1-3} have roused considerable interest in scientific research. Bacteria in this sessile form are 10 to 1000 fold more resistant than in the planktonic state\textsuperscript{4}, being an important virulence factor and one of the main challenges in clinical practice.

Marine-derived fungi are an important source and present a diversity of chemical compounds, comprising e.g. terpenoids, polyketides, nitrogenous compounds (alkaloids and peptides), and others\textsuperscript{5}, showing diverse biological activities such antimicrobial, anticancer, antioxidant and, anti-inflammatory effects.\textsuperscript{6-8} Despite intensive search for new molecules with remarkable activities against new diseases, few studies have investigated metabolites from marine fungi to combat pathogenic biofilms.\textsuperscript{9}

Recently, we have started a screening study to investigate the potential of fungi isolated from marine organisms collected at South Brazilian Coast, from the Arvoredo Island in the Arvoredo Biological Marine Reserve (Santa Catarina state, Brazil), against bacterial pathogenic biofilms.\textsuperscript{9} Among these fungi, we selected the ascomycete \textit{Penicillium} sp. F37, isolated from the sponge \textit{Axinella corrugata} to become the object of research for novel compounds active against \textit{Staphylococcus epidermidis} biofilm. We report here, for the first time, the isolation of a dipeptide \textit{cis-cyclo}(leucyl-tyrosyl) from marine \textit{Penicillium} sp. possessing a remarkable anti-staphylococcal biofilm activity. Its structure was characterized on the basis of spectroscopic methods including extensive 2D NMR and MS spectrometry. Furthermore, antibiofilm and antibacterial activities were evaluated by crystal violet method, turbidimetric assay and scanning electron microscopy.

The fungal strain was identified by its ITS region and the sequence was deposited under GenBank HE608803. The fungal strain was cultivated in 5 L Sabouraud broth, at 25 °C for 7 days. The cultures were filtered through cheesecloth, lyophilized (19.48 g) and resuspended in distilled water. The solution was extracted with ethyl acetate (5 × 100 mL) and the organic extract was vacuum-concentrated to yield a red-orange residue (350 mg). The extract showed activity in preventing biofilm formation of \textit{Staphylococcus epidermidis} strains promoting the search for these bioactive compounds. The extract was dissolved in MeOH (130 mg mL\textsuperscript{-1}) and subjected to fractionation by semi-preparative HPLC RP-C18 column and eluted by using a gradient of A) water:methanol (9:1) and B) water:methanol (1:19), which was started
isocratically at 80:20 for 5 min., increased to 0:100 by 30 min, and was held isocratically for 10 min. The purified fraction was obtained as colorless needles (6 mg), and its molecular formula was found to be C_{15}H_{21}N_{2}O_{3} by HREIMS data at [M+H]^+ m/z 277.1540; calculated: 277.3419. Analysis of the $^1$H and $^{13}$C NMR spectra obtained in CD$_3$OD-$d_6$ suggested a dipeptide structure due to the presence of two methine protons, two methylenes and two carbonyl carbons. Protons of a para-substituted aromatic ring were seen in the $^1$HNMR spectrum at $\delta_H$ 7.02 (2H, d, 8.5 Hz) and 6.76 (2H, d, 8.5 Hz), coupling with a methylene group with $\delta_H$ 3.18 (1H, dd, 4.0, 14 Hz) and 2.91 (1H, dd, 4.0, 14 Hz). This methylene moiety coupled with a proton at $\delta_H$ 4.22 (1H, m), suggesting their attachment to a hetero atom, and indicating a tyrosyl residue. A leucyl residue was identified by the presence of an isopropyl group at $\delta_H$ 0.79 (two methyl), a methine multiplet at $\delta_H$ 1.47, a methylene group at $\delta_H$ 0.30 (1H, ddd, 4.7, 12 Hz), and 1.08 (1H, ddd, 4.2, 12 Hz) and, a methine at $\delta_H$ 3.71 (1H, dd, 4.0, 10.3 Hz). In the $^1$H-$^1$H COSY spectrum (Figure 1) it was possible to observe the couplings of aromatic ring of the tyrosyl residue, the attachment of methylene protons to the CH-3 at $\delta_H$ 4.22 Hz and CH-6 at $\delta_H$ 3.71 Hz of diketopiperazine, also the coupling of the methyl protons with methine at $\delta_H$ 1.47 Hz and this isopropyl group with methylene protons at $\delta_H$ 1.08 and 0.30 Hz. These assignments were confirmed by carbon signals equivalent to two amide carbonyl seen in the $^{13}$C NMR spectrum ($\delta_C$ 169.7 and 168.2). The carbon signal of the nitrogen-bearing methine bounds ($\delta_C$ 56.91 and 53.52) were observed attached, each one having couplings to CH$_2$ carbons ($\delta_C$ 39.25 and 44.22). The remaining $^{13}$C NMR data indicated the presence of 1,4-disubstitute benzene ring ($\delta_C$ 131.98, 126.2 and, 115.94) with an oxygenated carbon ($\delta_C$ 156.98). The carbons of the isopropyl group ($\delta_C$ 24.07, 23.1 and, 20.98) confirmed the previous identification of a leucyl moiety. The signals of HMBC indicated the correlations of the methylene in the tyrosyl residue with C1' ($\delta_C$ 126.21) and C2' ($\delta_C$ 131.93) of the aromatic ring, with the diketopiperazine methine at $\delta_C$ 56.91, and the carbonyl ($\delta_C$ 168.29). The leucyl methine and methylene group HMBC correlations were observed with the carbonyl at $\delta_C$ 169.70 The cis configuration could be deduced by the methine resonances in the diketopiperazine ring at $\delta_H$ 3.71 and the resonances of the leucyl part at $\delta_H$ 0.30 and 1.08 which were downshifted, indicating the shielding effects of the aromatic ring. The data were also compared to NMR data published by Huang et al. and Kopple and Marr which revealing very similar spectral properties. The compound was therefore identified as cis-cyclo(leucyl-tyrosyl).
Cis-cyclo(leucyl-tyrosyl) has never been found to interfere with biofilm formation and we characterized further its activity upon *Staphylococcus epidermidis* ATCC 35984. As showed in Fig. 2, the isolated compound was capable to inhibit biofilm formation in the range of 0.25, 0.5 and 1.0 mg mL⁻¹ (60, 65 and 85%, respectively) without interference on bacterial growth. In the SEM images performed with and without the dipeptide (Fig. 3), an expressive amount of *S. epidermidis* bacterial clusters with strong bacterial attachment made of exopolysaccharide (EPS) (indicated by the arrows in Fig. 3A) matrix could be shown in the untreated biofilm. However, in the sample treated with 1.0 mg mL⁻¹ of the dipeptide only few cell aggregates with weakly EPS formation are visible (Fig. 3B).

Diketopiperazine has a broad range of biological activities, which include: antitumor, antiviral, antifungal, antibacterial and antihyperglycemic effects.¹³⁻¹⁷ Only some dipeptides from marine microbial sources were reported, e.g. 8-amino-[1,4]diazonane-2,5-dione and leucyl-4-hydroxyproline isolated from the marine-associated actinomycete *Streptomyces acrimycinii*¹⁸; the diketopiperazines cyclo(L-Phe-L-Pro), cyclo(L-Pro-L-Tyr), cyclo(L-Pro-L-Val), and bilains A-C from the Australian marine-associated *Penicillium bailaii*¹⁹; the diketopiperazines cyclo(D)-Pro-(D)-Phe, (D)-Pro-(D)-Leu,(D)-Pro-(D)-Val and a (D)-4-OH-Pro-(D)-Phe from marine bacteria associated with cultures of larvac of *Pecten maximus* molluscs, with activity against the pathogen *Vibrio anguillarum*²⁰ and, recently, three diketopiperazine alkaloids and eight analogues were isolated from the deep-ocean sediment fungus *Penicillium griseofulvum*.²¹ Many of these compounds have been reported to possess antimicrobial and biofilm modulating activities but cis-cyclo(leucyl-tyrosyl) was not reported to have any antibacterial or antibiofilm activities.²²

Our results are also important, since dipeptides have been related to be signaling molecules only in gram-negative bacteria. Some L-L-diketopiperazine have recently been know as quorum-sensing bacterial sensors as in *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Citrobacter freundii* and *Enterobacter agglomerans* that produce small and diffusible diketopiperazines which activate quorum-sensing systems with Lux-R like proteins.¹²,²³,²⁴, a finding, however, questioned by results of other authors.²⁵ Cyclo(L-Pro-L-Met) and cyclo(L-Pro-L-Tyr) for example, present remarkable importance since the cyclo(L-Pro-L-Met), produced by *E. coli*, stimulates the swarming motility of a *swrI* mutant of *Proteus mirabilis* as effectively as C4-homoserine lactone. In contrast, cyclo(L-Pro-L-Tyr) and other diketopiperazines antagonize the quorum-sensing-
regulated swarming of *Serratia liquefaciens*. The action of N-acyl homoserine lactones can be mimetized by diketopiperazines by interacting with LuxR proteins at, or near, the their binding site.

Nevertheless, a study demonstrated the production of the dipeptides cyclo(L-Phe-L-Pro) and cyclo(L-Tyr-L-Pro) by the gram-positive *Lactobacillus reuteri* RC-14 (vaginal isolate) that were able to repress the expression of the virulence factor TSST-1 of *Staphylococcus aureus* MN8 (prototype of menstrual toxic shock syndrome), showing dipeptides as signaling molecules, interfering with the quorum-sensing system *agr* of gram-positive bacteria.

The evidence that this mentioned 2,5-diketopiperazine is active against *S. epidermidis* biofilm formation is new. This information increase the range of possibilities to treat biofilm infections, pointing to an alternative to the known – and with emerging bacterial resistance - mechanisms of action of antibiotic. Both, the exploration of this activity as molecular mechanism of action and the evaluation of analogues by combinatorial chemistry is imminent. This class of molecules have revealed a variety of activities and the possibility of facile synthesis, where high yields could be obtained, makes them even more interesting.

**Acknowledgments**

CNPq-Brazil (Jovens Pesquisadores – Nanotecnologia), CNPq Universal 2009, Rede Nanobiotec CAPES-Brazil, and FAPERGS. Environmental licenses to collect samples are granted by CGEN (Ministério do Meio Ambiente) in application 020000.002820/2003-12. MS acknowledges also the scholarship from CNPq and DAAD. The authors thank the Centro de Microscopia Eletrônica (CME/UFRGS) for technical assistance in electron microscopy.

**Supplementary data**

Supplementary data associated with this article can be found, in the online version
References


**Fig. 1.** Correlations of 2D MNR of *cis-cyclo*(leucyl-tyrosyl). Bold line: $^1$H-$^1$H COSY correlation. Solid arrows: HMBC correlations.

**Fig 2.** Evaluation of antibiofilm and bacterial growth activities against *S. epidermidis*. Grey bars represents percentual of biofilm formation; grey squares represents bacterial growth. Results represent mean ± standard deviation of 3 experiments. (*) $p \leq 0.05$. 
Fig. 3. Scanning electron microscopy images of the *S. epidermidis* biofilm formation. A - Untreated sample (biofilm formation); B - Treated sample (cis-cyclo(leucyl-tyrosyl) [1.0 mg mL$^{-1}$]) observed in 24 hours of treatment. Scale bars: 1-22,000× magnification (insert in the images: 2-200× magnification, 3-800× magnification, and 4-9000× magnification). Solid arrows: biofilm matrix.