This is a pre- or post-print of an article published in
Scopel, M., Abraham, W.-R., Henriques, A.T., MacEdo, A.J.
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. Macedo^{1,3*}

¹Faculdade de Farmácia, Departamento de Produção de Matéria-Prima, Universidade Federal do Rio Grande do Sul, Av. Ipiranga, 2752, 90610-000, Porto Alegre, Brazil.

² Helmholtz 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, conjunctivitis¹⁻³ have roused considerable interest in scientific research. Bacteria in this sessile form are 10 to 1000 fold more resistant than in the planktonic state⁴, 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 others⁵, showing diverse biological activities such antimicrobial, anticancer, antioxidant and, anti-inflammatory effects.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.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.⁹ Among these fungi, we selected the ascomycete *Penicillium* sp. F37, isolated from the sponge *Axinella corrugata* to become the object of research for novel compounds active against *Staphylococcus epidermidis* biofilm. We report here, for the first time, the isolation of a dipeptide *cis-cyclo*(leucyl-tyrosyl) from marine *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 *Staphylococcus epidermidis* strains promoting the search for these bioactive compounds. The extract was dissolved in MeOH (130 mg mL⁻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 $C_{15}H_{21}N_2O_3$ by HREIMS data at $[M+H]^+ m/z$ 277.1540; calculated: 277.3419. Analysis of the 1H and, ^{13}C NMR spectra obtained in CD_3OD-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 1H NMR spectrum at δ_H 7.02 (2H, d, 8.5 Hz) and 6.76 (2H, d, 8.5 Hz), coupling with a methylene group with δ_H 3.18 (1H_a, dd, 4.0, 14 Hz) and 2.91 (1H_b, dd, 4.0, 14 Hz). This methylene moiety coupled with a proton at δ_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 δ_H 0.79 (two methyl), a methine multiplet at δ_H 1.47, a methylene group at δ_H 0.30 (1H_a, ddd, 4.7, 12 Hz), and 1.08 (1H_b, ddd, 4.2, 12 Hz) and, a methine at δ_H 3.71 (1H_a, dd, 4.0, 10.3 Hz). In the 1H - 1H 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 δ_H 4.22 Hz and CH-6 at δ_H 3.71 Hz of diketopiperazine, also the coupling of the methyl protons with methine at δ_H 1.47 Hz and this isopropyl group with methylene protons at δ_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 (δ_C 169.7 and 168.2). The carbon signal of the nitrogen-bearing methine bounds (δ_C 56.91 and 53.52) were observed attached, each one having couplings to CH_2 carbons (δ_C 39.25 and 44.22). The remaining ^{13}C NMR data indicated the presence of 1,4-disubstitute benzene ring (δ_C 131.98, 126.2 and, 115.94) with an oxygenated carbon (δ_C 156.98). The carbons of the isopropyl group (δ_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' (δ_C 126.21) and C2' (δ_C 131.93) of the aromatic ring, with the diketopiperazine methine at δ_C 56.91, and the carbonyl (δ_C 168.29). The leucyl methine and methylene group HMBC correlations were observed with the carbonyl at δ_C 169.70. The *cis* configuration could be deduced by the methine resonances in the diketopiperazine ring at δ_H 3.71 and the resonances of the leucyl part at δ_H 0.30 and 1.08 which were downshifted, indicating the shielding effects of the aromatic ring.¹⁰ The data were also compared to NMR data published by Huang et al.¹¹ and Kopple and Marr¹² 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 acrimycini*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 bailai*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 larvae 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 activities²².

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 diffusable 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*.²⁶ The action of N- acyl homoserine lactones can be mimetized by diketopiperazines by interacting with LuxR proteins at, or near, the their binding site.²³

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.²⁷

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 emmerging 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

5. Saleem, M.; Ali, M. S.; Hussain, S.; Jabbar, A.; Ashraf, M.; Lee, Y. L. *Nat. Prod. Rep.* **2007**, *24*, 1142.
6. Bhatnagar, I.; Kim, S.-K. *Mar. Drugs* **2010**, *8*, 2702.
7. Tarman, K.; Lindequist, U.; Wende, K.; Porzel, A.; Arnold, N.; Wessjohann, L. A. *Mar. Drugs* **2011**, *9*, 294.
8. Bhimba, B. V.; Shilpa, A. P. T.; Jose, G. M.; Franco, A. D. *Int. J. Univ. Pharm. Life Sci.* **2011**, *1*, 74.
9. Scopel, M. P.h.D. Thesis. Universidade Federal do Rio Grande do Sul, February 2012.
1. Donlan, R. M.; Costerton, J. W. *Clin. Microbiol. Revi.* **2002**, *15*, 167.
2. Moreau-Marquis, S., Stanton, B. A., O'Toole, G. A. *Pulm Pharmacol Ther.* **2008**, *21*, 595.
3. Brady, R. A.; Leid, J. G.; Calhoun, J. H.; Costerton, J. W.; Shirtliff, M. E. *FEMS Immunol. Med. Microbiol.* **2008**, *52*, 13.
4. Gilbert, P.; Allison, D. G.; McBain, A. J. *J. Appl. Microbiol. Symposium Suppl.* **2002**, *92*, 98S.
10. Wang, G.; Dai, S.; Chen, M.; Wu, H.; Xie, L.; Luo, X.; Li, X. *Chem. Nat. Comp.* **2010**, *46*, 583.
11. Huang, H.; She, Z.; Lin, Y.; Vrijmoed, L. L. P.; Lin, W. *J. Nat. Prod.* **2007**, *70*, 1696.
12. Kopple, K. D.; Marr, D. H. *J. Am. Chem. Soc.* **1967**, *89*, 6193.
13. Song, M. K.; Hwang, I. K.; Rosenthal, M. J.; Harris, D. M.; Yamaguchi, D. T.; Yip, I.; Go, V. L. W. *Exp. Biol. Med.* **2003**, *228*, 1338.
14. Brauns, S. C.; Milne, P.; Naudé, R.; De Venter, M. V. *Antic. Res.* **2004**, *24*, 1713.

- 191 15. Rhee, K. H. *Int. J. Antimicrob. Ag.* **2004**, 24, 423.
- 192 16. Xiao, Q.; Pei, D. *J. Med. Chem.* **2007**, 50, 3132.
- 193 17. Martins, M. B.; Carvalho, I. *Tetrahedron* **2007**, 63, 9923.
- 194 18. Hernández, I. L. C., Macedo, M. L., Berlinck, R. G. S., Ferreira, A. G., Godinho, M.
195 J. L. *J. Braz. Chem. Soc.* **2004**, 15, 441.
- 196 19. Capon, R. J.; Stewart, M.; Ratnayake, R.; Lacey, E.; Gill, J. H. *J. Nat. Prod.* **2007**,
197 70, 1746.
- 198 20. Fdhila, F.; Vázquez, V.; Sánchez, J. L.; Riguera, R. *J. Nat. Prod.* **2003**, 66, 1299.
- 199 21. Zhou, L.-N.; Zhu, T.-J.; Cai, S.-X.; Gu, Q.-Q.; Li, D.-H. *Helv. Chim. Acta* **2010**, 93,
200 1758.
- 201 22. de Carvalho, M. P.; Abraham, W.-R. *Curr. Med. Chem.* **2012**, 19, 3564.
- 202 23. Degrassi, G.; Aguilar, C.; Bosco, M.; Zahariev, S.; Pongor, S.; Venturi, V. *Curr.*
203 *Microbiol.* **2002**, 45, 250.
- 204 24. Ryan, R. P.; Dow, J. M. *Microbiol.* **2008**, 154, 1845.
- 205 25. Campbell, J.; Lin, Q.; Geske, G. D.; Blackwell, H. E. *ACS Chem. Biol.*, **2009**, 4,
206 1051.
- 207 26. Daniels, R.; Vanderleyden, J.; Michiels, J. *FEMS Microbiol. Rev.* **2004**, 28, 261.
- 208 27. Li, J.; Wang, W.; Xu, S. X.; Magarvey, N. A.; McCormick, J. K. *Proc. Nat. Acad.*
209 *Sci. USA.* **2011**, 108, 3360.

Fig. 1. Correlations of 2D MNR of *cis-cyclo*(leucyl-tyrosyl). Bold line: ^1H - ^1H 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 $\pm$ 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.