Wattana-Amorn, P., Charoenwongsa, W., Williams, C., Crump, M. P., & Apichaisataienchote, B. (2016). Antibacterial activity of cyclo(L-Pro-L-Tyr) and cyclo(D-Pro-L-Tyr) from *Streptomyces* sp. strain 22-4 against phytopathogenic bacteria. *Natural Product Reports*, 30(17), 1980-1983. <https://doi.org/10.1080/14786419.2015.1095747>

Other version

Link to published version (if available):
[10.1080/14786419.2015.1095747](https://doi.org/10.1080/14786419.2015.1095747)

[Link to publication record in Explore Bristol Research](#)
PDF-document

This is the author accepted manuscript (AAM). The final published version (version of record) is available online via Taylor and Francis at <http://dx.doi.org/10.1080/14786419.2015.1095747>. Please refer to any applicable terms of use of the publisher.

University of Bristol - Explore Bristol Research

General rights

This document is made available in accordance with publisher policies. Please cite only the published version using the reference above. Full terms of use are available:
<http://www.bristol.ac.uk/red/research-policy/pure/user-guides/ebr-terms/>

SUPPLEMENTARY MATERIAL

Antibacterial activity of cyclo(L-Pro-L-Tyr) and cyclo(D-Pro-L-Tyr) from *Streptomyces* sp. strain 22-4 against phytopathogenic bacteria

Pakorn Wattana-Amorn^{a,*}, Waranya Charoenwongsa^a, Christopher Williams^b, Matthew P. Crump^b and Busaya Apichaisataienchote^{c,*}

^a*Department of Chemistry, Center of Excellence for Innovation in Chemistry and Special Research Unit for Advanced Magnetic Resonance, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand*

^b*School of Chemistry, University of Bristol, Cantock's Close, Bristol BS8 1TS, United Kingdom*

^c*Department of Biotechnology, Faculty of Engineering and Industrial Technology, Silpakorn University, Nakhon Pathom 73000, Thailand*

** Corresponding author:*

Email: fscipwa@ku.ac.th

Tel: +66-2-562-5555 ext 2234

Fax: +66-2-562-5555 ext 2119

Email: busayaa@gmail.com

Tel: +66-34-219364 ext 25200

Fax: +66-34-219360

Abstract

Two bioactive cyclic dipeptides, cyclo(L-Pro-L-Tyr) and cyclo(D-Pro-L-Tyr), were isolated from the culture broth of *Streptomyces* sp. strain 22-4 and tested against three economically important plant pathogens, *Xanthomonas axonopodis* pv. citri, *Ralstonia solanacearum* and *Clavibacter michiganensis*. Both cyclic dipeptides were active against *X. axonopodis* pv. citri and *R. Solanacearum* with MIC of 31.25 µg/mL. No activity could be observed against *C. michiganensis*.

Keywords: cyclo(L-Pro- L-Tyr); cyclo(D-Pro-L-Tyr); *Streptomyces*; phytopathogenic bacteria

1. Experimental

1.1 Bacterial isolation and production of *Streptomyces* sp. strain 22-4

Streptomyces sp. strain 22-4 was isolated from rhizospheric soil samples of healthy asparagus from organic farms in Nakhon Pathom, Thailand. The seed culture of *S.* sp. strain 22-4 was grown in the production medium (10 L) containing 20 g/L potato starch, 10 g/L peptone, 3 g/L K_2HPO_4 , 0.01 g/L $FeSO_4 \cdot 7H_2O$ and 0.5 g/L $MgSO_4 \cdot 7H_2O$. After 9 days incubation at 30 °C with 200 rpm agitation, the culture broth was centrifuged to obtain the cell-free supernatant and subjected to further extraction and isolation. In order to confirm that both cyclo(L-Pro- L-Tyr) and cyclo(D-Pro- L-Tyr) were endogenously produced by *S.* sp. strain 22-4, the medium only was also prepared using the same approach prior for HPLC analysis.

1.2 Isolation of cyclo(L-Pro- L-Tyr) and cyclo(D-Pro- L-Tyr)

The 10 L of culture broth was passed through Diaion[®] HP-20 resin (250 g) and washed extensively with distilled water. The resin was subsequently eluted with 500 mL of 50% MeOH/H₂O and concentrated to dryness to obtain 13 g of crude extract. 5 g of the crude extract was further fractionated using a Sephadex LH-20 column. Each fraction was further purified using HPLC equipped with ACE C18 semi-preparative column (10 μ m, 10 $\times$ 250 mm, 3 mL/min) using a gradient of 5-40% MeOH/H₂O supplemented with 0.1% TFA for 45 min. The UV absorbance was monitored at 254, 280 and 360 nm respectively. From 5 g of the crude extract, 3 mg of mixture containing cyclo(L-Pro- L-Tyr) and cyclo(D-Pro- L-Tyr) was obtained. The ¹H and ¹³C chemical shifts of both compounds are shown below.

cyclo(L-Pro- L-Tyr) (A): ¹H NMR (CD₃OD, 600 MHz) δ_H 7.04 (2H, AA'BB', J = 8.4 Hz, H-12, H16), 6.71 (2H, AA'BB', J = 8.4 Hz, H-13, H15), 4.37 (1H, t, J = 4.6 Hz, H-3), 4.05 (1H, ddd, J = 10.8, 6.3, 1.4 Hz, H-6), 3.51-3.57 (1H, m, H-9), 3.34-3.38 (1H, m, H-9'), 3.09 (1H, dd, J = 14.2, 4.6 Hz, H-10), 3.03 (1H, dd, J = 14.2, 4.6 Hz, H-10'), 2.07-2.12 (1H, m, H7), 1.19-1.26 (1H, m, H7'), 1.78-1.83 (2H, m, H8); ¹³C NMR (CD₃OD, 150 MHz) δ_C 170.8 (C-5), 167.0 (C-2), 157.7 (C-14), 132.1 (C-12, C-16), 127.6 (C-11), 116.2 (C-13, C-15), 60.1 (C-6), 57.9 (C-3), 45.9 (C-9), 37.7 (C-10), 29.4 (C-7), 22.7 (C-8).

cyclo(D-Pro- L-Tyr) (B): ¹H NMR (CD₃OD, 600 MHz) δ_H 6.98 (2H, AA'BB', J = 8.4 Hz, H-12, H16), 6.72 (2H, AA'BB', J = 8.4 Hz, H-13, H15), 4.15 (1H, t, J = 4.6 Hz, H-3),

3.51-3.57 (1H, m, H-9), 3.29-3.33 (1H, m, H-9'), 3.11 (1H, dd, $J = 13.9, 4.6$ Hz, H-10), 2.88 (1H, dd, $J = 13.9, 4.6$ Hz, H-10'), 2.60 (1H, dd, $J = 10.3, 6.2$ Hz, H-6), 2.04-2.07 (1H, m, H7), 1.88-1.94 (1H, m, H8), 1.60-1.70 (1H, m, H7'), 1.62-1.67 (1H, m, H8'); ^{13}C NMR (CD_3OD , 150 MHz) δ_{C} 171.4 (C-5), 167.6 (C-2), 158.2 (C-14), 132.3 (C-12, C-16), 126.9 (C-11), 116.4 (C-13, C-15), 59.9 (C-3), 59.2 (C-6), 46.1 (C-9), 40.2 (C-10), 29.8 (C-7), 22.5 (C-8).

1.3 General experiments for structure determination

The structure of compounds dissolved in CD_3OD was elucidated using 1D (^1H , ^{13}C) and 2D NMR (COSY, HSQC, HMBC and TOCSY) NMR spectra. All spectra were recorded at 25 °C on an INOVA 400 and a cryoprobe equipped Varian VNMRs 600 spectrometers. For the high resolution mass spectrum, the data was collected on Bruker micrOTOF-Q III with atmospheric-pressure chemical ionisation (APCI).

1.4 Determination of the absolute configuration of amino acids

The mixture of cyclic dipeptides (1.5 mg) was hydrolysed with 6 M HCl (1 mL) at 120 °C for 24 h. The absolute configuration of amino acids was determined using Marfey's method modified from Bhushan and Brückner's protocol (Bhushan and Brückner 2007). The hydrolysed product was then dried and re-dissolved in water (100 μL) before 200 μL of Marfey's reagent in acetone (37 mM) was added to 50 μL of hydrolysed cyclic dipeptides. This was followed by addition of 40 μL of 1 M NaHCO_3 . The mixture was then heated with shaking at 45 °C 200 rpm for 1 h. When the solution was cooled to room temperature, 20 μL of 2 M HCl was added to the reaction mixture. The mixture was dried under vacuum and was subsequently dissolved in 200 μL of DMSO. The mixture was then analysed by HPLC using Phenomenex C18 column (10 μm , 3.90 $\times$ 300 mm, 1 mL/min) with a gradient of 5-40% $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ supplemented with 0.2% TFA over 60 min. The UV absorbance was detected at 340 nm. The non-treated hydrolysate with Marfey's reagent was prepared and analysed using the same approach as above. Each of the Marfey products was also confirmed by LC-MS.

1.5 Antibacterial assay

A mixture of cyclo(L-Pro-L-Tyr) and cyclo(D-Pro-L-Tyr) was isolated from the culture media which could not be separated using chromatographic methods, therefore synthetic cyclo(L-Pro-L-Tyr) and cyclo(D-Pro-L-Tyr) (Cellmano, china) (>95% purity) were used in the antibacterial assays against three plant pathogenic bacteria i.e. *X. axonopodis* pv. citri, *R. solanacearum* and *C. michiganensis*. These organisms were obtained from Institute of Phytomedicine, University of Hohenheim, the Federal Republic of Germany. Antibacterial activity was evaluated using the modified resazurin method (Sarker et al. 2007). A stock concentration of 1 mg/ml of the test compound and the antibiotics were prepared. All stock solutions of compounds and antibiotics were filtered-sterilised using a 0.2 μ m syringe filter. Two-fold serial dilutions of the antibiotics and compounds were prepared with nutrient broth to give concentrations from 0.12 to 500 μ g/mL. Thereafter 10 μ L of bacterial suspension (10^6 CFU/mL) was added to each well to achieve a concentration of 10^5 CFU/mL. The plate was incubated at 35 °C for 18-24 h. Finally, 10 % (v/v) of resazurin indicator solution was added. The plate was further incubated at 35 °C for 2 h and the colour change was assessed visually. Any colour changes from purple to pink or colourless were recorded as positive. The lowest concentration at which color change occurred was recorded as the MIC value. Triplicate sets of plates were maintained for each concentration for the test sample.

Figure S1. HR-MS spectrum of cyclo(L-Pro-L-Tyr) and cyclo(D-Pro-L-Tyr) mixture

Figure S2. Comparison of ^1H NMR spectra between (A) synthetic cyclo(L-Pro-L-Tyr), (B) synthetic cyclo(D-Pro-L-Tyr) and (C) cyclo(L-Pro-L-Tyr) and cyclo(D-Pro-L-Tyr) mixture isolated from *Streptomyces* sp. strain 22-4

Figure S3. ^{13}C NMR spectrum (CD_3OD , 600 MHz) cyclo(L-Pro-L-Tyr) (numbering with A) and cyclo(D-Pro-L-Tyr) (numbering with B) mixture

Figure S4. COSY spectrum (CD₃OD, 600 MHz) of cyclo(L-Pro-L-Tyr) (numbering with A) and cyclo(D-Pro-L-Tyr) (numbering with B) mixture

Figure S5. TOCSY spectrum (CD_3OD , 600 MHz) of cyclo(L-Pro-L-Tyr) (numbering with A) and cyclo(D-Pro-L-Tyr) (numbering with B) mixture

Figure S6. HSQC spectrum (CD₃OD, 600 MHz) of cyclo(L-Pro-L-Tyr) (numbering with A) and cyclo(D-Pro-L-Tyr) (numbering with B) mixture

Figure S7. HMBC spectrum (CD₃OD, 600 MHz) of cyclo(L-Pro-L-Tyr) (numbering with A) and cyclo(D-Pro-L-Tyr) (numbering with B) mixture

1 2 * 3 * 4
1 2 3

Figure S8. HPLC chromatograms of peptide hydrolysate before A) and after B) adding Marfey's reagent. Some products from incomplete hydrolysis or contamination reacted with Marfey's reagent can be observed in chromatogram as marked with *. C) HPLC chromatogram of Marfey's derivative of L-proline (1), D-proline (2), L-tyrosine (3) and D-tyrosine (4), numbers are indicated in the chromatogram. The UV absorbance was detected at 340 nm.

Bhushan R, Brückner H. 2004. Marfey's reagent for chiral amino acid analysis: A review. *Amino acids*. 27:231-247.

Sarker SD, Nahar L, Kumarasamy Y. 2007. Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals. *Methods*. 42:321-324.